

**NUTRITION AND REPRODUCTION IN THE
ECTOPARASITIC HONEY BEE (APIS SP.) MITE,
VARROA JACOBSONI**

DISSERTATION

**zur Erlangung des Grades eines Doktors
der Naturwissenschaften**

der

Fakultät für Biologie

der Eberhard-Karls-Universität Tübingen

vorgelegt von

NARESH CHANDRA TEWARSON

aus Pauri-Garhwal/Indien

1983

**NUTRITION AND REPRODUCTION IN THE
ECTOPARASITIC HONEY BEE (APIS SP.) MITE,
VARROA JACOBSONI**

DISSERTATION

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

der

Fakultät für Biologie

der Eberhard-Karls-Universität Tübingen

vorgelegt von

NARESH CHANDRA TEWARSON

aus Pauri-Garhwal/Indien

1983



Tag der mündlichen Prüfung: 29. Juli 1983

Dekan: Professor Dr. K.P. Hadeler

1. Berichterstatter: Professor Dr. W. Engels

2. Berichterstatter: Professor Dr. P. Wenk

ACKNOWLEDGEMENTS

This study was proposed by Prof. Dr. Wolf Engels who, from the beginning of my doctoral work in 1980 till its completion in 1983, supported me with many helpful suggestions and constructive criticism. I am grateful for and appreciative of his guidance.

Prof. Engels was also instrumental in arranging for contact with other laboratories. I extend thanks to Profs. Ruttner and Koeniger as well as their co-workers, Bee Research Institute at Oberursel, not only for providing me with the mites necessary for my work, but also for the hospitality which they showed to me during my research stays there. Thanks is also due to Prof. Peters and his co-workers at the Institute for Zoology III in Düsseldorf, who enabled me to perform special studies on gut ultrastructure. Special thanks goes to Prof. Bardele and the technical staff at the electron microscopy lab in the Institut of Zoology in Tübingen. Not only did they introduce me to basic EM techniques but they also proved a continual source of ready help when advice concerning various problems was sought.

Within the group of Developmental Biology, I thank all the scientific and technical staff members as well as my co-candidates who very kindly helped me during my entire stay. I would like to mention the following people personally: beekeepers Geert Staemmler and Franz Hertl, and the secretarial staff, Catherine Messer and Jonathan Whitlock.

My research stay in Germany was made possible by a Ph.D scholarship granted by the Ökumenisches Studienwerk, Bochum. I am very thankful for this opportunity to broaden my knowledge during these last 5 years in Germany.

Finally, I would like to express my deepest love to my wife, Sneha and children without whose help and encouragement this study would never have been completed.

'SCIENCE' is a body of knowledge obtained by regerous use of the scientific methods, which assumes that events in the observable world follow sets of rules or generalization that, once dicovered, can be used to predict subsequent events.

..... David J. Horn.

CONTENTS

	Page no.
A. INTRODUCTION	1
A.1.1. Social insects (honey bees, <u>Apis</u> sps.).	1
A.1.2. Natural history and distribution of <u>Apis</u> .	2
A.1.3. Honey bee diseases and predators.	2
A.2.1. Parasitic mites of honey bees.	3
A.2.2.1. Distribution of <u>Varroa jacobsoni</u> .	6
A.2.2.2. Parasitic damages in <u>A.mellifera</u> colonies.	8
A.2.2.3. Diagnosis of varroatosis.	8
A.2.2.4. Control of varroatosis.	10
A.2.2.5. Life history of the <u>Varroa</u> mite.	11
A.2.3.1. Biology of host-parasitic relationship.	13
A.2.3.2. Aims and scope of this study.	14
B. MATERIAL and METHODS	17
I NUTRITION IN VARROA	
B.1.1. <u>Quantitative determination of the amount of <u>Apis</u> hemolymph volume sucked by female mite, <u>Varroa jacobsoni</u>.</u>	17
B.1.1.1. Volume of hemolymph in <u>Apis</u> drone and worker L ₅ larvae by Isotope dilution method.	17
B.1.1.2. Honey bee hemolymph meal volume sucked by female <u>Varroa</u> under different conditions.	13
B.1.2. <u>Immunological and electrophoretical detection of <u>Apis</u> (host) hemolymph proteins in the hemolymph of <u>Varroa</u> (parasite).</u>	19
B.1.2.1. Production of antisera.	19
B.1.2.2. Radial immunodiffusion in agarose gel.	20
B.1.2.3. Ouchterlony double-immunodiffusion.	21
B.1.2.4. Immuno-electrophoresis.	21
B.1.2.5. Two diamensional Tandem-cross-immunoelectrophoresis	22
B.1.2.6. SDS-polyacylamide slab gel electrophoresis.	23
B.1.3. <u>Ingestion of <u>Apis</u> drone and worker larval food by reproductive female mites.</u>	24
B.1.3.1. Radial-immunodiffusion.	25

B.1.4.	<u>Undigested uptake of bovine serum albumen (BSA) in the hemolymph by Varroa.</u>	24
B.1.4.1.	Injections of albumen in the nursing honey bees and collection of hemolymph.	24
B.1.4.2.	Radial-immunodiffusion.	25
B.1.4.3.	Ouchterlony immunodiffusion.	25
B.1.5.	<u>Proteolytic-enzyme assays of the midgut proteases of Varroa.</u>	25
B.1.5.1.	Photopaper test.	26
B.1.5.2.	Proteolytic activity in polyacrylamide slab gels.	26
B.1.5.3.	Overall protease activity with synthetic substrates.	27
B.1.5.4.	Specification of <u>Varroa</u> proteases.	27
B.1.5.5.	Identification of <u>Varroa</u> exopeptidase.	28
B.1.5.6.	Protease inhibitor in the <u>Apis</u> hemolymph.	28
B.1.6.	<u>Histological and immunohistochemical studies of midgut and excretory tubes of Varroa.</u>	28
B.1.6.1.	Fixation and embedding for histological studies.	28
B.1.6.2.	Fixation and embedding for histochemistry (PAP)	29
B.1.6.3.	Section cutting and post treatment of semi and ultra-thin sections.	29
B.1.6.4.	The unlabelled-antibody-enzyme method (PAP) for immunohistochemical studies of midgut of <u>Varroa</u> .	29
II REPRODUCTION IN <u>VARROA</u>		
B.2.1.	Preference of <u>Apis</u> drone larvae to worker larvae by <u>Varroa</u> female for reproduction.	31
B.2.1.1.	Biotest with <u>Apis</u> drone and worker larvae by <u>Varroa</u> female for reproduction.	31
B.2.1.2.	Kairomone test in-vitro and in natural bee colonies.	31
B.2.2.	<u>Immunological and electrophoretical detection of Apis hemolymph proteins in the eggs of Varroa.</u>	33
B.2.2.1.	Ouchterlony immunodiffusion in agarose gels.	34
B.2.2.2.	Immuno-electrophoresis.	34
B.2.2.3.	Two dimensional Tandem-cross-immuno-electrophoresis.	34
B.2.2.4.	SDS-polyacrylamide slab gel electrophoresis.	34

B.2.3.	<u>Immunohistochemical (PAP) localization of Apis hemolymph antigens in the vitellogenic oocytes of Varroa.</u>	35
B.2.4.	<u>Vitellogenesis in Varroa.</u>	35
B.2.4.1.	Detection of <u>Apis</u> and <u>Varroa</u> hemolymph proteins in the hemolymph of <u>Varroa</u> by SDS-PAGE.	35
B.2.4.2.	Identification of foreign (<u>Apis</u>) and <u>Varroa</u> 's own hemolymph proteins as its vitellogenins by SDS-PAGE.	36
B.2.4.3.	Light and electron microscopic studies of the functional morphology of oogenesis in <u>Varroa</u>	36
C.	RESULTS	38
	I NUTRITION IN VARROA	38
C.1.1.	<u>Quantitative determination of the amount of Apis hemolymph volume sucked by female mite, Varroa jacobsoni.</u>	38
C.1.1.1.	Volume of hemolymph in <u>Apis</u> drone and worker L ₅ larvae as determined by the isotope dilution method.	38
C.1.1.2.	Honey bee hemolymph meal volume sucked by <u>Varroa</u> female under different conditions.	38
C.1.2.	<u>Immunological and electrophoretical detection of Apis hemolymph proteins in the hemolymph of Varroa.</u>	40
C.1.2.1.	Production of antisera.	40
C.1.2.2.	Radial immunodiffusion.	40
C.1.2.3.	Ouchterlony double immunodiffusion.	40
C.1.2.4.	Immunoelectrophoresis.	41
C.1.2.5.	Two dimensional Tandem-cross-electrophoresis.	41
C.1.2.6.	SDS-polyacrylamide slab gel electrophoresis (PAGE)	42
C.1.3.	<u>Ingestion of Apis drone and worker larval food by reproductive female mites.</u>	43
C.1.3.1.	Radial immunodiffusion.	43
C.1.4.	<u>Undigested uptake of BSA in the hemolymph of Varroa mite.</u>	43
C.1.4.1.	Injections in nursing bees.	43
C.1.4.2.	Radial immunodiffusion	43
C.1.4.3.	Ouchterlony double immunodiffusion.	43

C.1.5.	<u>Proteolytic enzymes of Varroa.</u>	44
C.1.5.1.	Photopaper test.	44
C.1.5.2.	Proteolytic activity in polyacrylamide slab gels.	44
C.1.5.3.	Overall protease activity with synthetic substrates.	45
C.1.5.4.	Specification of <u>Varroa</u> protease.	45
C.1.5.5.	Identification of <u>Varroa</u> exopeptidases.	45
C.1.5.6.	Protease inhibitor in <u>Apis</u> hemolymph.	45
C.1.6.	<u>Histological and immunohistochemical studies of midgut and excretory tubes of Varroa.</u>	46
C.1.6.4.	The unlabelled antibody-enzyme method (PAP) for immunohistochemical studies of midgut.	48
	II. REPRODUCTION IN VARROA	48
C.2.1.	<u>Preference of drone larvae to worker larvae by reproductive Varroa female.</u>	48
C.2.1.1.	Bio-test with drone and worker larvae.	48
C.2.1.2.	Kairomone test in-vitro and in natural bee colonies.	49
C.2.2.	<u>Immunological and electrophoretical detection of host proteins in the eggs of Varroa.</u>	50
C.2.2.1.	Ouchterlony double immunodiffusion.	50
C.2.2.2.	Immuno-electrophoresis.	50
C.2.2.3.	Two dimensional Tandem-cross-immuno-electrophoresis.	51
C.2.2.4.	SDS-polyacrylamide electrophoresis.	51
C.2.3.	<u>Immunohistochemical localization of host antigens in the vitellogenic oocytes of Varroa.</u>	52
C.2.4.	<u>Vitellogenesis in Varroa</u>	53
C.2.4.1.	Detection of <u>Apis</u> and <u>Varroa</u> hemolymph proteins in <u>Varroa's</u> hemolymph.	53
C.2.4.2.	Identification of foreign (<u>Apis</u>) and <u>Varroa's</u> own hemolymph proteins as its vitellogenins by SDS-PAGE.	53
C.2.4.3.	Light and electron microscopical studies of the functional morphology of oogenesis in <u>Varroa</u> .	54
D.	DISCUSSION	61
	I. NUTRITION IN <u>VARROA</u>	61
D.1.1.	The physiology of nutrition in <u>Varroa</u> .	61
D.1.2.	Functional morphology of midgut and excretory tubes	65

and immunocytochemistry of midgut.

II. REPRODUCTION IN VARROA 66

D.2.1. Parasitic adaptations of Varroa in reproduction. 66

D.2.2. Vitellogenesis in Varroa. 67

D.3. The significance of nutrition in the reproduction
of Varroa 70

E. SUMMARY

F. LITERATURE

G. CURRICULUM VITAE

A. INTRODUCTION

A.1.1.Social insects (Honey bee, Apis sp.)

Four out of every five animal species are insects. The unique diversity of insects has direct economical and social consequences for humans. Not only do insects cause damage to forests and agriculture, but they also carry diseases which claim millions of lives each year; all of which has had a major impact on human history and economic development. Nevertheless, insects can be beneficial as well. Production of honey and bees wax, silk, lac, food, and other articles; their function as pollinators, in biology, and in medicine as experimental animals are just some examples of their usefulness.

Among the beneficial insects, honey bees are the most celebrated. Honey was perhaps the first sweet substance tasted by primitive man. Besides honey, other honey bee products (viz., wax, royal jelly, venom, and propolis) have been found to be of great use to mankind in day-to-day life as well as in the medical, cosmetic, munition, and candle industries to name a few.

Throughout the world today, honey bees are the most important of the insect pollinators. Their capacity as efficient pollinators lies in the fact that, although they visit a variety of flowers, they restrict each foraging trip to a single plant species (flower constancy, VON FRISCH,1950). Because of their ready availability, the honey bee is the only species which a fruit, vegetable, or oil seed grower can rent in quantity for the purpose of pollinating his monocultures.

From the innumerable species of solitary insects, there are tens of thousands of species of bees, wasps, ants and termites which employ social structure to solve the ecological problems which are usually faced by a single insect. They are among the great achievements of organic evolution. The colonies of insects are often called super-organisms because they show a number of social phenomena which can be compared to the physiological properties of organs and tissues.

Among the social insects, appreciable amounts of honey and wax can only be obtained from bee colonies belonging to the genus- Apis, commonly known as the honey bee. Although there are other kinds of

bees, such as Meliponini (stingless bees) and bumble bees (Bombini), the quantity of honey collected by them is much less and of little economic importance. All bees belong to the family- Apidae, which in turn includes two highly social tribes- Meliponini (stingless bees) and Apini (Apis).

A.1.2. Natural history and distribution of honey bees (Apis sps.)

There are some 20,000 species of bees out of which about 500 are social. Only 4 species of genus- Apis (true honey bees) are found in the world today. 3 of these 4 species occur in abundance in a wild state in tropical southeast Asia which is their natural home. These are the giant honey bees (Apis dorsata), the dwarf honey bee (A. florea), and eastern honey bee (A. cerana). The 4th species is the european honey bee (A. mellifera) whose native home is Europe and Africa.

The european honey bee and eastern honey bee, which are physically similar (the latter being a little smaller in size), have attracted the special attention of man because of their remarkable nature. The reason being their capacity to store large amounts of accessible honey. Secondly, due to their habit of nesting in dark crevices, hollow tree trunks, ect., they can be easily induced to nest in movable containers or hives.

During the past few hundred years, the european honey bee (A. mellifera) has been established and distributed almost throughout the world, especially in America, Australia, and New Zealand with success. On the African continent, there are several naturally occurring strains of A. mellifera. The eastern honey bee, A. cerana or indica, is restricted to S.E. Asia, China, east USSR and Japan and has to a certain extent been replaced by A. mellifera, particularly in the temperate zones of these regions due to the efforts of beekeepers.

A. Honey bee diseases, predators, and parasites.

Before domestication, honey bees had more enemies in the primitive forest than now, when beekeepers have provided them with wooden

houses to their advantage. Nevertheless, many enemies of domesticated bees remain. Some of the diseases of which we know today were identified even thousands of years ago by Aristotle (384-322 B.C.). The following is a summary of bee diseases and predators:

- Viruses: Paralysis virus, chronic bee-paralysis virus associate, sacbrood virus, black queen cell virus, filamentous virus and bee virus-Y, bee virus-X, cloudy wing particles, acute bee paralysis virus, slow paralysis virus, Kashmir bee virus, Arkansas bee virus, Egyptian bee virus, Apis iridescent virus.
- Bacteria: American foulbrood, European foulbrood, secondary bacteria (Bacterium eurydice) and others.
- Fungi: Chalkbrood, stonebrood, yeasts, and others.
- Protozoans: Nosema apis, Malpighamoeba mellificae, Gregarines (such as Leidyne etc.) and flagellates (such as Leptomonas apis).
- Insects and nematodes: Hymenopterans, Dipterans (such as Senotainia tricuspidis etc.), Braula coeca, Coleopterans (such as larvae of the beetle Meloe spp.). Strepsipterans and Lepidopterans (such as Galleria mellonella), Nematodes (such as Mermis nigriscens and Agamormes spp.).
- Diseases of yet uncertain origin and noninfectious diseases.
- Diseases of unknown origin (e.g. The Isle of White disease).
- Plant poisons.

A.2.1. Parasitic mites (Acari) of honey bees

According to a recent review of mite pests of honey bees by DE JONG et al. (1982), about 83 species of Acari (mites), either found on the bees themselves or in the hives, have been reported to be associated with honey bees (Apis spp.). Many of the mites have no economic importance as their host and habitat are other than Apis. Many are plant-inhabiting and phoretic on honey bees or occasional visitors of hives. Four species of mites are closely associated with

Apis sps. and are of economic importance:

- Acarapis woodi.
- Tropilaelaps clarae.
- Euvarroa sinhai.
- Varroa jacobsoni.

Acarapis woodi (Rennie) :

The causative agent of 'acarine disease' in honey bees was first described by RENNIE et al. (1921) as Tarsonemus woodi but was later renamed by HIRST (1921) as Acarapis woodi. It belongs to the Trombidiformes and infests the trachea that leads from the first pair of prothoracic spiracles of adult bees. Occasionally, when the infestation is heavy, they may infect the air sacs.

There are 2 other external sps. of this mite - A. externus and A. dorsalis. These 3 related species are morphologically distinct and infest a particular part of the bee. A. externus is localized in the area behind the head on the ventral side of the neck in the adult bee. A. dorsalis lives on the thorax in a groove between the mesoscutum and mesoscutellum and also at the base of the wings, on the fore wings, and the fore parts of the abdomen. From the studies of BRÜSI-PAL (1934) it appears that all the species of Acarapis feed on hemolymph.

These two external species are distributed world wide, however, A. woodi occurs almost throughout the world with the exception of the USA, Canada, Australia, and New Zealand. The damage done by this mite depends upon the environment and condition of the bee colonies. It is no longer considered to be an alarming disease of honey bees. Fumigation of colonies with chlorobenzilate, sulphur impregnated fuel, specific acaricides such as 'Demite' and Folbex ect., have proved to be effective in controlling these mites.

Tropilaelaps clarae :

This mite was discovered by DALFINADO and BAKER (1961) in the Philippines on A. mellifera colonies. Like Varroa jacobsoni, it is a large (0.96-0.55 mm), fast moving, light reddish brown ectoparasite of honey bee brood and adults. It is the original parasite of

A. dorsata and was found in the Philippines and in India. It has been observed, however, that the mite imparts much greater damage to recently introduced A. mellifera colonies in these countries. The adult mite and its protonymph and deutonymph feed on hemolymph of bee brood and adult bees. The symptoms of the infection of this mite are very much like those of Varroa jacobsoni infestations. It results in irregular brood patterns, malformation of the larvae, pupae, and adult emerging bees, and abdomen and wing deformities (ATWAL AND GOYAL, 1971). The other two species, A. florea and A. cerana, were not found to be infested by this mite (ATWAL and DHALIWAL, 1969). The mite is restricted to tropical Asia. Chlorobenzilate and powdered sulphur were used successfully in controlling Tropilaelaps clarae. Dusting the brood nest with powdered sulphur proved to be very successful in eliminating the mite from A. mellifera colonies (ATWAL and GOYAL, 1971).

Euvarroa sinhai :

This ectoparasitic mite was found by DALFINADO and BAKER (1974) in the dwarf honey bee A. florea colonies in India. It is a large mite (measuring about 1.04-1.00 mm), brown, and pear-shaped. The life cycle of this mite and Varroa jacobsoni are bionomically similar with the exception that Euvarroa sinhai infests only drone brood. All the nymphal instars and adult females feed by sucking on the hemolymph of the bees. The adult males are not able to feed because the chelicerae are modified as sperm transfer organs similar to that of Varroa. However, it is not yet certain whether adult phoretic females also feed on adult bees. The mite has not been reported to infest the commercial bees, A. mellifera, A. cerana and A. dorsata.

Varroa jacobsoni :

Acarid, Varroa jacobsoni, was found for the first time on the bodies of A. cerana on the Indonesian Island of Java by the entomologist Edward Jacobson, and was described in detail by OUDEMAN (1904). The mite was redescribed by GUNTHER (1951) in Sumatra as Myrmozercon reidi. However, in 1963, the name was again changed to Varroa jacobsoni by DALFINADO. Varroa jacobsoni is a relatively

large ectoparasitic mite of A. cerana which is its original host. Before man began experimenting with bees for maximum gain, the two economically important species, A. mellifera and A. cerana, were geographically separated. After A. mellifera was introduced at several places in tropical southeast Asia, both species of honey bees came in closer contact. The mite, Varroa jacobsoni, found a more suitable 'ecological niche' in A. mellifera colonies and started invading them at an alarming rate. The host-parasite balance, attained during the long association with A. cerana, failed in the case of A. mellifera, the colonies of which suffered great losses (RITTER, 1981).

The mite and its developing stages parasitize all of the honey bee stages, including late larval, prepupal and pupal, adult drone and worker bees, and feed on their hemolymph. Varroa's preference for drone larvae for reproduction (GROBOV, 1977), results in a low number of drones available in areas where A. mellifera colonies are of low density. Thus, the virgin queen remains unseminated. As a result of this, the colonies die. Transport of honey bees from infested areas to mite free areas has resulted in most severe infestations. This is also true of migratory beekeeping, swarms, flying workers, and drones. In West Germany, varroaosis spreads at a natural speed of between 2.5 and 3 km per year (RUTTNER and RITTER, 1980).

A.2.2.1. Distribution of Varroa jacobsoni.

As stated before, Varroa jacobsoni is an established ectoparasitic mite of eastern honey bee, A. cerana. From 1904 to 1960, the mite thrived unnoticed in A. cerana colonies in the far eastern regions of Asia. The warm climate of the equatorial and tropical regions, provide suitable conditions for the propagation of this mite throughout the year. The intensity of infection increases tremendously because the complete life cycle of Varroa takes only 8-10 days for female and 6-7 days for male mites (GROBOV, 1977; SMIRNOV, 1978).

In the early 1960's, Varroa was discovered in A. mellifera colonies in the Philippines just as it was being established

(DALFINADO, 1963). The switching-over of hosts from eastern to european bees was perhaps initiated by close contact between both species and also by beekeepers who practiced the introduction of A. cerana brood to A. mellifera colonies in other Asian countries for the purpose of strengthening them (SHABANOV et al., 1978). Consequently, the european colonies of A. mellifera in many places succumbed to the Asian mite (MORSE, 1969).

In 1964, the mite was discovered first in the far east of the Soviet Union in the Primorje region in A. mellifera colonies. After that, the mite was identified and described in south China and later in India, Pakistan, Indonesia, PR korea, Japan, and other parts of China. Between the years of 1965 and 1970, the parasite was distributed in the far east of the USSR and eventually everywhere in Russia, where it was responsible for enormous losses of bee colonies. During 1967, the mite was reported in Bulgaria the reason being the import of the infested queens. In Rumania, Varroa was found in 1975. It was supposed that the mite entered in these two countries 3 to 5 years before its discovery (NEDEALKOV, 1977). In 1978, the mite was discovered in Tunesia and in 1976 in Libya where the bees were imported from Rumania and Bulgaria respectively without quarantine measures.

All the countries of eastern Europe are now infested with Varroa. In western Germany, a separate infestation started accidentally near Frankfurt. A. cerana (apparently infested) were imported from Pakistan for research purposes in 1971. Perhaps the mites again changed hosts and were discovered in 1977 in the A. mellifera colonies (RUTTNER and RITTER, 1980).

In 1971, Paraguay imported A. mellifera colonies (infested) from Japan where the mite was already established in the bees. This was the first entry of Varroa into South America (DE JONG and GONÇALVES, 1981). At present, North America, Canada, New Zealand, Australia, and the most western parts of Europe, are the only areas which seem to be free of Varroatosis. However, the intensity with which the mite has spread throughout the world within the last decade, indicates that as long as a host is available it has the potential to reach anywhere.

A.2.2.2. Parasitic damages in A. mellifera colonies.

Great losses have been reported in the equatorial and tropical regions where A. mellifera was introduced. In the Philippines, about 1,000 colonies vanished, an estimated 55,000 colonies were lost in the USSR, in China and Japan there have been total losses of colonies of A. mellifera in some apiaries (DE JONG et al., 1982).

As the mite and its developmental stages suck the hemolymph from late larval, prepupal, pupal and adult stages of the bee, the haemocyte counts in the hemolymph sinks about 30% (in the adult bee), causing the protein titre to go down to dangerously low levels (SMIRNOW, 1978). Besides this, the infested bees become easily prone to microorganisms which enter through the wound into the hemolymph. Nevertheless, in adult bees the damage is less severe because in this case the mite is mainly phoretic or habitates during the winter break in breeding only to survive. On the other hand, severe damage is done by heavy infestations of sealed brood. When such bees emerge, they have either crippled legs and wings or shortened abdomen. Such bees are barely viable and eventually the colony is weakened and dies (DE JONG et al., 1982).

In the first year of infection (1-10 mites/colony), Varroa-tosis does not become apparent. In the second year of infestation (maybe 100 mites), the colony still appears healthy except for a few crippled bees (removed by the worker bees) which may be seen near the flight board of the hive. In the third year (perhaps over 1,000 mites), no apparent clinical symptoms appear except for a few crippled bees. However, during the fourth year (more than 1 mite infests each cell), the colony becomes very weak and may succumb altogether in autumn because so many dying larvae, pupae, and emerging bees cannot be replaced by the few healthy bees. In such mass infections, the bees leave the hive in swarms and die due to cold or other factors. In winter, the death rate is at its highest due to heavy infestations (GROBOV, 1977; RITTER, 1981).

A.2.2.3. Diagnosis of Varroa-tosis

The major part of the life cycle of Varroa is spent in the bee

brood cells where the mite is not easily noticed. It is also not easy to identify Varroa on adult bees because they hide under the abdominal sclerites and also tend to be initially confused with the ectoparasitic bee lice, Braula sps. However, with a little care, this parasite can be distinguished from Varroa; Braula is longer than it is wide and being an insect possesses three pairs of legs.

Several diagnostic methods are available for determining the presence and degree of Varroa infestations.

a- Examination of the hive floor

At times the mites die and fall from the emerging bees and can be collected from the hive floor. This method has proved to be successful in autumn and winter when the mortality of the mites is at its highest. To achieve this, the floor of the hive is covered with a sheet of thick white paper or plastic mounted on a wooden frame. It is fitted 6mm above with a gauze (3 mm. mesh) to keep the bees from falling down. This is checked for mites at intervals with either the naked eye or by floatation method of hive debris (BREM, 1980). This method can be combined with the treating of the colonies with various acaricides.

b- Examining the brood.

The capped brood cells are opened with the help of a pair of forceps. The larvae or pupae are removed and checked for Varroa as is the interior of the cells. When the drone brood is available, then examination of the outer edges of the brood frames is the most effective and time saving method, because the mite prefers the drone brood. Although this method is more trouble, it gives more accurate estimations.

c- Examination of the adult bees.

About 200-300 bees are collected, preferably from the open brood combs, and immersed in a detergent solution and shaken for 1.5 hrs. This is then strained through a white cloth after which the mites can be checked on the cloth after separating out the bees. The use of gasoline or hexan increases the efficacy of this method 85 to 100 % (RITTER, 1980 b).

A.2.2.4. Control of Varroaosis.

The close association of the mite with bees during development, makes it difficult to control the mite chemically. Also, the contamination of honey and wax limits further use of chemotherapeutic agents. Some of the mites are inaccessible as they remain sealed within the bee brood. This is especially true in the temperate zones where there is a very short broodless period in winter. The present methods of chemical control, which are effective and satisfactory at all, tend only to reduce the number of mites. Researchers have tested a vast variety of acaricides in one country or certain area with success, only to find that the same chemical was ineffective in another. This was perhaps due to different methods used, the environmental conditions, and the situation of the bee colonies under question.

A. Chemical control.

Phenothiazine, which is an insecticide and fungicide used for plant protection, and chlorobenzilate (Folbex, Ciba-Geigy) which is an acaricide used for 'Acarine disease' have been used extensively; the latter being the only acaricide permitted to be used in W. Germany with little or no residues. In Japan, resistance to Phenothiazine was reported (SMIRNOW and SCHERNOW, 1976). Chlorobenzilate does not kill all the mites and is therefore not an effective chemotherapeutic agent.

A Rumanian product, Sinacar, which consists of powdered sugar and various chemicals such as chloropropylate, bromopropylate, ect., showed a 95% effectiveness in that country, but in W. Germany, was less potent (RITTER, 1981) and in South America was totally ineffective (STORT et al., 1981).

Varostan (Bayer), a Japanese product found very effective in Japan, caused high bee mortality when tried in W. Germany (RITTER, 1981). There has been a list of chemicals such as malathion, denikoroper, formic acid, thymol, chlorodymeform hydrochloride (K-79) ect. which have been extensively used and which show both advantages and disadvantages in controlling the mite population. The intensive

efforts of several workers to discover an effective agent, seem to be failing. At present, Folbex-Forte, another product of Ciba-Geigy, seems to show promise. Its active ingredient is a contact poison, bromopropylate, which has been used for controlling spider mites in vegetables. It is being used in W. Germany in the form of smoker strips and has been shown to kill 90% of the Varroa mites and is well tolerated by the bees (RITTER, 1981). However, the effectiveness is yet to be seen in other places of the world.

B. Biological control.

a-Brood removal.

Varroa prefers drone brood, if available, to worker brood. The capped drone brood in individual colonies is removed and destroyed. This method destroys a majority of the reproductive female mites. In practice, this method is not feasible because of the great deal of labour involved. Sometimes, all of the capped brood are taken to an incubator. After they hatch, the bees are checked for mites and returned to the hives (SCHULZ and KOENIGER, 1980; RUTTNER and KOENIGER, 1980).

b- Heat treatment.

The mite cannot remain attached to the bodies of the bees at 46-48°C. This fact was developed into a method of detaching the mites from bees in Russia and Japan, the latter even receiving a patent for a hot air blower used for this purpose. Many bees die, however, and the treatment is moreover expensive (RITTER, 1981). The untiring efforts and fantasies of beekeepers and researchers have not as yet reached a satisfactory level of success, perhaps because of the limited knowledge of the biology of Varroa and its host-parasite relationship with the bee.

A.2.2.5. Life history of Varroa mite.

The ectoparasitic mite Varroa jacobsoni belongs to:

Phylum- Arthropoda

Sub Phylum- Chelicerata

Class-	Arachnida
Order-	Acari
Sub order-	Parasitiformes
Family-	Dermanicidae (previously Gamasidae)
Sub family-	Varroinae (including <u>Euvarroa sinhai</u> , an ectoparasite of <u>Apis florea</u>)
Genus-	<u>Varroa</u>
Species-	<u>jacobsoni</u> (Oudemans, 1904)

Varroa jacobsoni (as seen in the Raster Electron Micrographs is a large, dorsoventrally flattened, reddish-brown, oval (wider than long) mite. The female is 1.045-1.098 mm. long, 1.515-1.580 mm. wide, and is covered with thick cuticular covering with bristles. It has 4 pairs of short, stumpy, legs with suckers on the tarsi of each leg. The chelicerae of the female are modified into a stabbing sucking type, with which the mite drills a hole in the intersegmental membrane of the honey bee. The mite sucks the hemolymph from the adult bees, often from under the abdominal sclerites or between the thorax or ventral side of the thorax (SMIRNOW, 1978). The adult male Varroa is small and almost round with a greyish-white or yellowish color. Its body is 0.75-0.832 mm. long. The cuticular covering is weakly sclerotized. The chelicerae are modified for sperm transfer, hence, the males cannot feed and die soon after copulation.

The previously inseminated reproductive female mite enters the brood cell before the larva (5 days old) in the cell is capped. Two to five eggs (whitish and 0.5 mm in diameter) are laid, one at the time, on the walls of the cells. The embryo develops in 24 hrs. but remains inside the egg shell. After 48 hrs. of egg-laying, a six-legged protonymph hatches and feeds actively for 48 hrs. after which it molts into an eight-legged deutonymph which also actively feeds on the hemolymph of the pupae and looks similar to the adult mite except for its white color. After about 3 days, the deutonymph molts into imago which still have a lighter color than the mother mite. The complete development of the female mite takes 8-10 days and that of the male mite about 6-7 days. After copulation within the still capped brood cells, the male dies. The female mite leaves the

cell with the emerging bee.

SCHULZ (1983) was able to observe the egg-laying frequency of the Varroa mite by marking the female reproductive mite with dyes. Accordingly, he noticed that 78% of the mites go into the egg-laying phase only once, 18% twice, and 4% repeat it 3 times in their life. He also reported that the average number of daughter mites (progeny) in a worker cell was 1.8-1.9 per mite and in the drone cell, 2.2-2.4 per mite. It was seen in Varroa, that the female contains two types of embryos which indicates that it has the haplo-diploid system of sex determination or arrhenotokous type (STEINER et al., 1982 ; DE RUIJTER, 1983). It has been noticed that the first egg laid by Varroa always produces a female mite, the second always a male, and the rest of the eggs develop into female mites (DE RUIJTER, 1983).

The number of Varroa progeny is greater in male cells than in female Apis brood cells, because the worker bees take 21 days and drones 23-24 days to completely emerge. In worker cells, the emerging female mites from the last fourth and especially the fifth egg do not have enough time to mature and to copulate with the male. By the time the worker bees emerge, the uncopulated mites die. On the other hand, almost all of the emerging mites in the male cells can copulate and remain viable to produce progeny.

The female mites live for about 2 months in the summer and 5-8 months in the winter, during which they are inactive and stay attached to the winter bees (GROBOV, 1977; SMIRNOW, 1978; LANGHE, 1977).

A.2.3.1. Biology and host-parasite relationship.

The sudden outbreak of varroatosis in Apis mellifera colonies raised panic in the world. Beekeepers, bee pathologists, and veterinarians tried their best to control the disease but unfortunately a precise knowledge of the biology and host-parasite relationship of the bees and mites was lacking. Varroatosis thus remained the greatest threat to the bee industry in the world. This problem has been more acute in countries where bee breeding is highly developed

or in developing countries where bee breeding is being practiced in order to improve and modernize the bee industry. Consequently, few effective control methods have been developed. They are either too costly, tedious, or in some cases hazardous to the worker involved in beekeeping. Many of the acaricides can be dangerous to the consumers of honey and wax as well.

On the basis of previous experiences and failures, it was believed that a systemic acaricide would be the most effective (RUTTNER, 1980; TOSCHKOFF et al., 1980). In fact, TOSCHKOFF et al., (1978) suggested that an agent be developed which would reach the mite and its developmental stages in the capped brood cells sucking the hemolymph of the host. These systemic agents, when mixed with sugar syrup and fed to the bees, are quickly dispersed in the bee colony by means of trophallaxis. The agent reaches the adult bee through the hemolymph and the larvae through the larval food. Through the larvae or pupae in the sealed cells, enough substance reaches the nymphal stages or adult mites in the cells to kill them.

The use of a systemic acaricide such as chlordimeform-hydrochloride (K-79) in 47 bee colonies showed that 46 colonies became Varroa free after the treatment. That gives an elimination rate of 97.8% (RUTTNER, 1980). Such an effective control method requires a proper knowledge of the biology and the host-parasite relationship which is unfortunately still lacking. The Varroa mite lives in Apis cerana colonies without imparting any economic damage. The data concerning such a host-parasite balance are scanty. KOENIGER et al. (1981) while working with A. cerana, observed that Varroa enters worker and drone cells but lays eggs only in the drone cells. This is yet to be confirmed in other areas under different conditions.

A.2.3.2. Aims and scope of this study.

Varroa is notorious for causing the dreadful disease of the honey bee, varroaosis, and for its astonishing reproductive capacity and rate of spread. This is due to the perfect synchronization of its reproductive period with that of the developing stages of the honey bee, while sealed in the capped brood cells where they

enjoy optimal conditions for reproduction and development. However, the rather short period available for the mite to complete its reproduction and development in this atmosphere indicates a high degree of adaptation. In fact, there are many popular accounts of their propensity to overwhelm and destroy A. mellifera colonies. Unfortunately, the available information concerning its biology and host-parasite relationship is not sufficient. The facts just mentioned have barely drawn the attention of scientists occupied in this field of research. The present study is aimed at revealing some of the more important phases of the life cycle of Varroa jacobsoni.

The literature available about the nutrition of the mite is rather insufficient. SMIRNOW (1978) weighed hungry and fed Varroa females within 2 hrs. of feeding and calculated the hemolymph meal volume. MORITZ (1981) fed syrup containing ^{32}P to bees and measured the average radioactivity within 24 hrs. for young and adult mites. However, there are many discrepancies in both calculations. They might be due partly to the different methodology used. Besides this, the experiment lacks a precise description of the developmental and physiological status of host as well as the parasite itself.

The exact meal volume and frequency of feeding ect. are of great importance for determining systemic control methods in which chemotherapeutics are introduced to Varroa through the hemolymph of the host. In the present study, defined stages of host and parasite were used to determine the meal volume consumed by the mite and the total volume of host blood available for the mite in the host larvae by the isotope dilution method. Although the host hemolymph is the only food available to Varroa and its nymphal stages, studies over the fate of host blood in Varroa have not as yet caught the attention of the physiologists.

There are, on the other hand, a couple of accounts concerning the functional morphology which go in other directions. POLYAKOW et al. (1975) focused their studies on the locomotary and holding mechanisms of the mite with the help of electron microscopy. SADOW (1976) using light microscopy, studied the alimentary and excretory system

of the mite. LANGE et al. (1976) investigated at the same time the tracheal system of Varroa. SMIRNOWA et al. (1978) using light microscopy, probed the internal anatomy and lastly, IONESCU-VARO and SUCIU (1978) performed preliminary studies on the anatomy and histochemistry of the mite. The highlight of their study was the discovery of the spermatheca in the female Varroa.

A comprehensive account regarding the reproduction and its functional morphology in Varroa is still lacking. WITTE (1974) studied the oogenesis in Erythraeides (Acari: Trombidiformes) and MOTHES and SEITZ (1981) gave an account of the functional morphology of reproductive organs and oogenesis in a plant parasitic mite, Tetranychus urticae (Acari: family- Tetranychidae). There are a few studies on spiders (Aranae) and ticks (Ixodoidea). However, fine structural studies of the blood sucking Arthropodes are rare.

From the standpoint of evolutionary adaptation, the parasite-host relationship between Varroa and Apis should have reached a balance because during its life cycle, especially the phase of reproduction, the mite is in perfect harmony with the constant conditions provided by developing bee brood in the sealed cells. Although, as mentioned before, our knowledge of varroatosis in the original host species, A. cerana, is very scanty, suggestions about significant differences of the mite's reproduction in colonies of its secondary host, A. mellifera, are valuable. In order to understand this, detailed studies of nutrition, reproduction, and nutritive adaptations associated with reproduction are needed to answer many questions.

The present work has been focused on the above aspects. These studies should help us to understand the importance of the host (Apis) hemolymph and the role played by this special diet in the reproduction of the Varroa mite. This has also significance in the formation of newly developing systems for the control of varroatosis (systemic agent) and monitoring the population dynamics (reproductive quotient) of the mite.

B. MATERIAL & METHODS

I NUTRITION IN VARROA.

B.1.1. Quantification of the amount of Apis hemolymph volume sucked by a female mite, Varroa jacobsoni.

B.1.1.1. Volume of hemolymph in Apis drone and worker late larvae as determined by the isotope-dilution method.

The isotope-dilution method was used to measure the total hemolymph volume of bee larvae and the meal volume consumed by female Varroa mites. In this technique, a tracer molecule (^3H -Inulin), which is known to have low metabolism in Arthropodes, was administered to the bee larvae. About 25 drone and worker larvae were collected from freshly capped brood cells of Apis mellifera carnica colonies. The injections of the isotope were prepared by dissolving 1 mg of ^3H Inulin (sp. activity 1.1 mCi/mg, code TRA 324, Amersham, Buckinghamshire) in 5 ml of insect ringer and further diluted to obtain 6,000 to 10,000 cpm/ μl of the tracer solution aliquotes. 1 μl of this solution was injected into the dorsal blood vessel of the larvae with the help of glass microcapillaries pulled into fine tips. The injected larvae were kept at 35°C with a relative humidity of 70-80 %. Hemolymph (20 μl or less) was collected at different time intervals (5-30 min.) by making an incision on the dorsal blood vessel of the larvae. Larval hemolymph was mixed with 100 μl of tissue solubilizer (didodocyl dimethyl ammoniumhydroxide, Serva) and 5 ml scintillation fluid (aquasol). 20 μl of tracer solution aliquotes were also treated in the same manner. Radioactivity was measured in the liquid scintillation counter (Packard-tricarb with internal standard) and calibrated for 1 μl of solution. Hemolymph volume of late larvae was calculated using the formula:

$$\text{Volume of hemolymph } (\mu\text{l}) \text{ in the } \underline{\text{Apis}} \text{ larvae} = \frac{\text{cpm injected} / \mu\text{l}}{\text{cpm extracted} / \mu\text{l}}$$

(cpm values for the calculation were obtained by drawing a curve

taking cpm/ μ l against the time in minutes and the average of those values which lie in the plateau when the tracer molecule was evenly distributed in the hemolymph of the larvae.

B.1.1.2. Honey bee hemolymph volume sucked by a Varroa female mite under different conditions.

About 90 drone and worker late larvae and eight day old bees were collected. The same number of mites were also collected from late larvae, prepupae and pupae, and imaginal bee stages and were grouped accordingly. Before the experiment, a group of mites was kept without food for up to 48 hrs.

The isotope-dilution method described under B.1.1.1. was also used in this experiment to determine the meal volume of the Varroa female. 1 μ l of tracer solution was injected into the larvae. The nursing stage bees were injected intraabdominally through the inter-segmental membrane between the first abdominal segments on the lateral side. Both the unfed and fed mites were allowed to feed on the injected bee stages and they were stored at 35°C with 80-90% rel. humidity in a dark incubator to simulate the natural environment within the hive's brood nest.

After different time intervals (45 min. to 48 hrs) of feeding, mites were collected and each was homogenized in 100 μ l of tissue solubilizer (didodocyl dimethyl ammoniumhydroxide, Serva). 5 ml of scintillation fluid (aquasol) was added to it and the radioactivity was measured. 20 μ l or less of the hemolymph was collected from larvae and nursing stage bees previously injected with tracer molecule. Tissue solubilizer and scintillation fluid were added as before and radioactivity was measured and calibrated for 1 μ l hemolymph.

The mite meal volume was calculated as follows:

$$\frac{\text{Apis hemolymph in } \underline{\text{Varroa}} (\mu\text{l})}{\text{Apis hemolymph volume (in the beginning } \mu\text{l})} = \frac{\text{(a)}}{\text{(b)}} \times \frac{\text{\% of } \underline{\text{Apis}} \text{ hemolymph in } \underline{\text{Varroa}}}{\text{\% of } \underline{\text{Apis}} \text{ hemolymph in } \underline{\text{Varroa}}}$$

Where,

$$\text{(a)} = \text{Apis hemolymph volume} = \frac{\text{cpm injected} / \mu\text{l}}{\text{cpm extracted} / \mu\text{l} + \text{cpm in } \underline{\text{Varroa}}}$$

$$(b) = \% \text{ of Apis hemolymph in the mite} = \frac{\text{cpm in Varroa}}{\text{cpm injected in Apis}} \times 100$$

For maximum accuracy, the dried excrement of Varroa was collected and dissolved in 100 μ l of tissue solubilizer and 5 ml aqasol was added before measuring the radioactivity. To avoid error, the activity measured was added to corresponding Varroa samples.

B.1.2. Immunological and electrophoretical detection of Apis (host) hemolymph proteins in the hemolymph of Varroa (parasite)

B.1.2.1. Production of antisera

(a) Collection of hemolymph from Apis stages.

Antisera directed against the honey bee hemolymph proteins were prepared to follow the fate of host (Apis) hemolymph proteins in Varroa. For immunization, hemolymph was collected from late larval, prepupal, and pupal stages of drone honey bees. This was done by making an incision on the mid-dorsal blood vessel of these stages and collecting hemolymph in capillary pipettes (0.01 ml). The samples were immediately frozen at -20°C .

One sample of hemolymph of prepupae and two extra separated protein fractions of hemolymph of worker bees (H_1 and H_3b , Engels, 1972) were used from the laboratory stock for immunizing rabbits.

(b) Immunization.

Rabbits were immunized by injecting 500 μ l of hemolymph aliquotes mixed with 1 ml of sterilized 0.9 % NaCl solution and 1 ml of complete Freund's Adjuvant. In the case of drone samples, 500 μ l of hemolymph contained 1:1:1 ratio of late larval, prepupal, and pupal hemolymph. This combination of drone hemolymph was used while keeping in mind that these bee stages correspond to the active phase of oogenesis and egg-laying in Varroa and that the mite prefers drone larvae to worker larvae (RUTTNER et al., 1980a; RUTTNER and KOENIGER, 1980).

Before immunization, the rabbits were controlled for and pre-existing antibodies to bee proteins by means of an O-Test. Injections for immunization were given subcutaneously on the back of the rabbits. Three extra booster injections of 2.5 ml each of antigen

solution were given at weekly intervals. The antibody titre after the second and third booster injections was checked by the Radial-immunodiffusion method of MANCINI (1965).

c) Collection of antisera.

After a week of third or last booster doses, the antibody titre reached a satisfactory level. Blood was collected from the veins of the ear pinnae of the rabbit 5-6 times at two day intervals. The blood was left to coagulate at room temperature and later centrifuged at 2,000 g for 5 min. and the supernatant was collected with Pasteur pipettes. Once more the blood was centrifuged at 3,000 g for 10 min and the remaining serum was collected. Samples were stored at -20°C .

B.1.2.2. Radial-immunodiffusion in agarose gel.

a) Hemolymph collection from Varroa mite.

One of the most tedious aspects of this undertaking was to collect the hemolymph from such a tiny mite with a very thick cuticular body covering. The hemolymph samples were collected from mites narcotized with CO_2 as well as partly from the frozen mites. As instruments for this procedure, glass microcapillaries (5 μl) were pulled into very fine tips. Their other end was attached to a micromanipulator syringe to exert a mild suction force. Under the powerful stereomicroscope, the mites were carefully pierced by capillary tips from the ventral side into the interscutal membrane on the corners of metapodal shields and at times under genitoventral plates. Microsuctional force was maneuvered to suck the mite's hemolymph. Sometimes, 0.2 μl of insect ringer was carefully injected into its hemocoel before the collection. About 0.10-0.20 μl of hemolymph could be collected from an adult female Varroa mite. It was stored at -20°C after pooling.

b) Radial-immunodiffusion (MANCINI, 1965).

1% agarose gel was impregnated with 5% antiserum against Apis drone hemolymph mixture of late larval, prepupal, and pupal stages.

5 μ l of hemolymph diluted 1:2 with Barbitol buffer pH 8.6 and more in the case of Varroa was applied to antigen wells in the agarose (fig.4). The gel was incubated for 48 hrs. in a sealed damp chamber at 6-8°C. The non-precipitated proteins were washed out with 0.9% NaCl solution. The slides were pressed twice with distilled water and dried at 60°C. They were stained with Comassie brilliant blue-R 250 (Serva) for 10 min. and destained with the same solution without the dye. They were dried and photographed.

B.1.2.3. Ouchterlony-double immunodiffusion (OUCHTERLONY, 1948).

1% agarose dissolved in Barbitol buffer pH 8.6 was casted on microscopical slides. 5 mm antibody and 3 mm antigen wells were punched on agarose and were filled with anti-Apis worker (AA), drone mixture (AD), hemolymph protein fraction (AH₁), and vitellogenin (AV) sera. The middle antibody well was filled with 8 μ l of Varroa hemolymph (sample arrangement was reversed from normal method). All the samples were diluted with buffer (Barbitol pH 8.6). The rest of the steps follow those for Radial-immunodiffusion described under B.1.2.2. (b). Fig. 6(a) shows immunodiffusion where 7 μ l of anti-drone mixture serum was used in the antibody well 5 μ l hemolymph of Varroa female, protonymph, deutonymph, egg homogenate in Barbitol buffer pH 8.6 and drone's late larval, prepupal, and pupal mixture hemolymph was filled in the antigen wells. Fig. 6 (b) illustrates a parallel diffusion where all the conditions are the same as in Fig. 6 (a) except that anti-drone mixture serum, anti-worker serum, and, in place of drone hemolymph mixture antigen, hemolymph of worker (nursing stage) bees were used.

B.1.2.4. Immuno-electrophoresis.

The micromethod of immunoelectrophoresis (SCHEIDEGGER, 1955), was run on 70 x 52 mm glass plates (Fig. 7). On these plates, 1.5 mm $\varnothing$ antigen wells and 2 mm x 50 mm reservoirs parallel to electrophoretic direction were punched on 1% agarose in Barbitol buffer pH 8.6. Electrophoresis was carried out in Kohn Shandon electrophoretic chambers (England) at 8°C for 45 min, 20 mA / 2 plates at

145 Volts. 5 μ l of hemolymph samples of Apis and Varroa were applied to antigen wells after starting electrophoresis to minimize the diffusion.

After the electrophoresis, the antibody reservoirs were loaded with 30 μ l of anti-drone serum and anti-worker serum. Immunodiffusion was carried out in a humid chamber for 24 hrs..at 8°C. The rest of the steps correspond to those of the radial-immunodiffusion reported under B.1.2.2. (b)

For better comparison, the sample arrangement was modified by combining the methods of SCHEIDEGGER(1955) and WILLIAMS and WEMYSS (1961).

B.1.2.5. Two dimensional Tandem-cross-immunoelectrophoresis (Krøll, 1973).

Cross immunoelectrophoresis of two antigen samples in the same run with the antigen wells punched in such a manner as to be compared with precipitin peaks formed from the respective samples which fuse to produce double peaks. Hence, this process facilitates a direct comparison of two different cross immunoelectrophoretic patterns.

Glass plates of 70 x 52 mm were covered with 5.8 ml buffered 1% agarose (Barbital pH 8.6). Two antigen wells were punched 11 mm from the longer and 50 mm from the broader side keeping a gap of 5 to 7 mm between the two holes. 5 μ l of hemolymph (Apis and Varroa samples) was loaded in the wells. After diffusion, the wells were closed with a drop of agarose solution. Immediately, the plates were connected to the buffer with filter paper wicks and run two plates at a time in the Kohn Shandon electrophoresis chamber. The running conditions were 12 mA / plate for 45 to 60 min. at 8°C for the first dimension. Leaving 15 mm agarose strip of sample slide, the rest of the agarose was removed. This empty part of the plate was casted with 0.5-1% antisera impregnated in 1% agarose. Second dimensional electrophoresis was run (8 mA / plate) for 16-20 hrs. at 8°C. After this, non-precipitated proteins were washed with 0.9% NaCl solution for 3 hrs. and the gels were stained with Comassie blue as described for the radial-immunodiffusion in B.1.2.2.

B.1.2.6. SDS-polyacrylamide slab gel electrophoresis.

In contrast to classical protein separation techniques, the SDS-polyacrylamide electrophoresis of LAEMMLI (1970) with its discontinuous buffer system has proved to be far more advantageous for the separation of bee and Varroa hemolymph proteins in two ways. First of all it gives a very high resolution unknown in other types of electrophoreses. Secondly, molecular weights of the separated proteins can easily be measured by comparing them with standard proteins.

a) Preparation of gels.

In this study, a complete LKB- 2001 vertical gel electrophoresis unit (Sweden) was used. 12 to 15 % polyacrylamide (acrylamide especially purified for electrophoresis, BDH, England) separating gels were prepared with cross linker, N,N' methylenebisacrylamide (Serva) in Tris-(Hydroxymethyle-) aminomethane base (Merck). Sodium dodecyl sulphate (SDS, BDH) buffer pH 8.8. Polymerization was performed by initiator TEMED (N,N,N',N'- Tetramethylethylenediamin, Serva) and catalizer, ammonium persulphate (Bio rad).

After an hour, 3-5% stacking gels were casted, this time using Tris-HCl (Sigma) with SDS-buffer pH 6.8. 4mm sample wells were made by using combs. Gels were left overnight for complete polymerization.

b) Sample preparation.

A protein concentration of about 0.2-2 mg / ml was found suitable in the sample buffer solution. Hemolymph was centrifuged at 3,500 g for 10 min. 0.5 μ l of it was mixed with 10 μ l of sample buffer to get a concentration of 1:20. The sample buffer included Tris-HCl buffer pH 6.2, extra SDS, glycerol (Merck) and 2-Mercaptoethanol (Merck). The buffered samples were dissociated completely by boiling at 100⁰C for 2-5 min. and immediately chilled at -20⁰C. A small amount of tracking dye (Bromophenol blue) was added before applying the samples.

c) Conditions for electrophoresis

5-15 μ l of the samples were applied with the help of a microsyringe

and two gels were run parallel in the LKB electrophoresis chamber for 12 - 15 hrs. with a constant current of 30 mA, 500 Volts and 2.5 Watts at room temperature.

d) Staining and Scanning of the gels.

Gels were stained with 0.1 % Comassie-Brilliant blue-R-250 (Serva) in methanol and acetic acid for 1 hr. under constant shaking and destained with the same solution without stain. The ready gels were photographed and scanned with gel scanner, Chromoscan-200 (Joyce-Loeble, England) by keeping the ratio of 1:1 between bands and peaks.

B.1.3.1. Radial-immunodiffusion

As DE JONG et al. (1982) observed, Varroa also ingests a small amount of worker or drone jelly after entering the brood cells, just before capping.

About 15 mites were collected from the larval food in the bottom of the cells. They were very carefully washed ten times with distilled water to avoid any possibility of external contamination during homogenization in Barbital buffer (pH 8.6). The homogenate was centrifuged twice at 4,000 g and supernatant was used as antigen against the anti jelly-Royal serum for immunodiffusion. In this experiment, 1 % agarose impregnated with 2 % anti-Royal jelly serum (AR) from the lab stock, was casted to carry out the radial-immunodiffusion described under B.1.2.2. 1.5 mm wells were punched in the gels and 2 ul of drone larval food, royal jelly, and Varroa homogenate were applied. After allowing the diffusion for 48 hrs., the gels were treated and stained with Comassie blue as before.

B.1.4. Undigested uptake of bovine serum albumin (BSA) in the hemolymph by Varroa.

B.1.4.1. Injection of albumin into the bees and collection of hemolymph.

8 - 10 ul of 25 % bovine serum albumin in insect ringer was

injected into the abdomen of each nursing stage honey bee, Apis mellifera carnica. 30 adult Varroa females were collected from nurse stage bees from the infested laboratory colonies. The BSA injected bees carrying 2-3 Varroa mites each were caged under hive conditions (32°C, 60 % rel. humidity) in an incubator. After 12, 24, 36, and 48 hrs. the mites were detached and their hemolymph was collected in microcapillaries with great care so as not to puncture their alimentary system as described under B.1.2.2. (a). After narcotizing the injected bees on which the mites had fed, the hemolymph was collected in micropipettes by making a small incision on the mid-dorsal blood vessel. Both the samples were deep frozen at -20°C and stored at the same temperature until the immunodiffusion was carried out.

B.1.4.2. Radial-immunodiffusion.

1 % agarose gel impregnated with 10 % anti-albumin serum (Aa) (Gibco, Grand Island, N.Y.) was casted on slides (see B.1.2.2.). 2.5 µl hemolymph in Barbitol buffer ph 8.6 from previously injected nurse stage worker bees with BSA, Varroa mites which had sucked on these bees, and BSA solution in the buffer was loaded in the antigen wells. After diffusion for 48 hrs. the gels were pressed, stained and photographed as described above.

B.1.4.3. Ouchterlony double-immunodiffusion.

This method has already been described under B.1.2.3. 5 µl of anti-bovine serum albumin (Aa) was applied to the antibody well. 2 µl of hemolymph from the injected bees (A), mites which sucked on these bees (v) and BSA dissolved in buffer were applied twice to the antigen wells. The 1 % agarose gel in Barbitol buffer pH 8.6. was incubated for 48 hrs. for complete formation of antibody-antigen complex. The gels were treated in the same way as described in B.1.2.3. above.

B.1.5. Proteolytic enzyme-assays of midgut of Varroa.

The small size and thick cuticular covering of the mites makes it almost impossible to dissect out the midgut for enzymatic

assays. Hence, a homogenate of 30-40 whole mites was made in ice cold 0.2. M Tris-HCl buffer pH 8.2. It was centrifuged at 11.000 g for 20 min. and supernatant was collected. Before the enzyme studies were performed it was centrifuged again.

B.1.5.1. Photopaper test

Normal white photopapers were exposed to light and developed into a black color. They were soaked in 0.2. M Tris-HCl buffer pH 8.2. A few drops of mite homogenate supernatant (along with prctease test mixture (Serva) in buffer for control) were applied to the surface of these pretreated photopapers. They were incubated in a damp chamber inside an incubator at 37°C for 1-4 hrs. After the end of the incubation period, the papers were checked for proteolytic degradation of the albumin coating on them.

B.1.5.2. Proteolytic activity in polyacrylamide gels.

With the help of this method, a direct detection of protease enzyme in gels is possible. 6-12 % polyacrylamide gels were casted with the help of horizontal slab gel unit. The sample slits were filled with 3-10 µl of Varroa homogenate supernatant, protease test mixture (Protease K, code 33752, Serva) and midgut homogenates of Sarcophaga fly for comparison. After joining the gel with filter papers to the buffer tanks, the electrophoresis was run in the LKB-Bromma-2117 multiphore unit (Sweden) with cooling water temperature 4°C for 2-4 hrs. at 30 mA/ 10 samples in glycine-NaOH-HCl buffer with different pH values. Afterwards, the gels were incubated in a bath containing buffered medium (pH 8.6-10.6) for 45-360 min. at 37°C. The incubation medium included either hemoglobin or bovine serum albumin as substrates. The gels were fixed, washed and stained with Coomassie blue (see B.1.2.6.). The sites of proteolytic degradation of substrate on the gels are revealed as weakly or non-colored zones against an evenly blue colored background (negative staining). The colored background is due to the protein substrate which diffuses into the polyacrylamide gels during incubation. Variations in the pH (8.6-10.6), substrate concentrations

(0.2-2 %) and the quantity of mite homogenate (3-10 μ l) were performed to obtain optimal resolution.

B.1.5.3. Overall activity with synthetic substrate.

Two synthetic substrates, Azocasein (DAHLMAN, et al., 1978) and Hide Powder Azure (Serva) were used to check the overall protease activity. To measure the color reaction, substrates were dissolved in 0.2 M Tris-HCl buffer pH 8.2 to get 1 % substrate concentrations. However, Hide Powder Azure is an only partially soluble substrate. Therefore, incubation had to be done under constant shaking. 30 μ l of Hide Powder Azure and Azocasein supernatant was mixed with 20 μ l of mite extract and incubated for 1-3 hrs. at 37°C. The reaction was stopped by adding 500 μ l of 8 % TCA (Trichloro acetic acid) and keeping it at 8°C for 30 min. Then, the solution was centrifuged at 3,000 g for 10 min. and the extinction of the TCA soluble supernatant was measured at 366 nm for Azocasein and at 578 nm for Hide Powder Azure. (table 2). Blanks were obtained by adding TCA to the substrates before adding Varroa extract. For the qualitative comparison, a Protease test mixture (Protease-K, code 33752, Serva) was treated in the same manner.

B.1.5.4. Specification of Varroa proteases.

To specify the Varroa proteases either in exopeptidases or endopeptidases, the following synthetic substrates were used:

A. N- Glutaryl-L-phenylalanine-4-Nitroanilid (GPNA)
(ERLANGER et al., 1966).

B. N- Benzoyl-L-arginine-4-Nitroanilid (BAPA) (ERLANGER et al.,
(1961).

C. L-Alanine-4-Nitroanilid (ANA) (JANZ, 1976).

The three substrates above were dissolved in 0.2 M Tris-HCl buffer pH 8.2 to obtain 1 % optimal substrate concentration. The mixtures of 50 μ l of substrate solution (A,B, and C) and 20 μ l of Varroa homogenate were incubated for 1-3 hrs. at 37°C. The indication of proteolytic breakdown appears as a yellowish tinge due to

the liberated nitroaniline After 3 hrs. of incubation the reaction was stopped by adding 2 ml of 8 % TCA to the solution and storing it at 8°C for 1 hr. This was centrifuged at 3,000 g and in the supernatant, extinction was measured at 405 nm (Table 3 and 4). For comparison, blanks were substituted and treated as described under B.1.5.3.

B.1.5.5. Identification of Varroa exopeptidase.

Following substrates were used to check if the mite's protease (exopeptidase) contained carboxypeptidase-A or -B like activity:

1. Hippuryl-lysine for carboxydase-B.
2. Hippuryl-phenylalanine for carboxydase-A.
3. L-Alanine-p-Nitroanilid was also used to detect the aminopeptidase-like activity.

The substrates and enzymes (in the case of the mite, the homogenate) were treated in the same manner as described under B.1.5.3. However, extinction for hippuryl-lysine and hippuryl-phenylalanine was monitored at 254 nm and for L-Alanine-p-Nitroanilid at 405 nm (see table 4).

B.1.5.6. Protease inhibitor in the hemolymph of Apis.

50 µl of different nitroanilid substrates and 20 µl of protease test mixture (Serva) were mixed with 5 µl of Apis worker hemolymph. Inhibitory effect of the bee hemolymph was seen by comparing the extinction with sample without Apis hemolymph at 405 nm.

B.1.6. Histological and immunohistochemical studies of midgut excretory tubes of Varroa.

B.1.6.1. Fixation and embedding for histology.

About 100 female Varroa mites were collected from the infested Apis mellifera carnica colonies. The mites were narcotized with CO₂ and fixed in 2.5 % glutaraldehyde buffered with either 0.05 M phosphate buffer pH 7.2 or 0.1 M cacodylate buffer pH 7.3 for 2-5

hrs. and post fixed in 2 % osmium tetroxide (OsO_4) for 1 hr. Because of the thick cuticular covering of the mites, the legs were removed or a small slit was made in the cuticle for improving the ability of the fixatives for penetration into the tissue. After dehydration, the mites were embedded in Epon-812.

B.1.6.2. Fixation and embedding for histochemistry (PAP).

The mites were fixed in 2.5 % glutaraldehyde in 0.05 M phosphate buffer pH 7.2 containing 4 % sucrose for 2 hrs. at 4°C . The mites were transferred to 0.05 M phosphate buffer pH 7.2 containing 8 % sucrose for 1.5 hrs. at 4°C , during which the buffer was changed 6 times. Here also the legs of the mites were removed for better infiltration of the fixatives. The mites were washed in buffer overnight at 4°C and then post fixed with 1 % OsO_4 buffered with the phosphate buffer as before for 2 hrs. at 0°C . Block staining was done with 70 % acetone saturated with zinc-uranyl acetate for 2 hrs. at room temperature in the dark. The mites were then embedded in Epon as before.

B.1.6.3. Section cutting and post treatment.

Semithin sections ($1-3\ \mu\text{m}$) were cut on LKB Ultramicrotome and stained with alkaline Toluidin blue-0 (Merck). They were photographed with a Zeiss Photomicroscope. Ultrathin sections ($50-70\ \text{nm}$) after contrasting in uranyl acetate for 30 min. and lead citrate for 6 min., were studied and photographed with a transmission electron microscope S-102 Siemens at 80 kV.

B.1.6.4. The unlabelled antibody-enzyme method (PAP) for immunocytochemistry.

Immunocytochemical studies of semithin sections were carried out by means of unlabelled antibody-enzyme method of STERNBERGER (1974) and STERNBERGER et al. (1970) modified with the technical parameters of ERLANDSEN et al. (1979) and THOMSON et al. (1980). The scheme of the technique is illustrated in fig. 1. Semithin PAP Epon sections ($0.25-1.0\ \mu\text{m}$) mounted on microscopical slides were treated

as follows:

a) Pretreatment of plastic for immunostaining:

Pretreatment of the sections to enhance immunostaining and for removal of plastic resin from the sections was done by dipping the slides containing sections in ethanol saturated with NaOH for 5 min.

b) Blocking of endogenous peroxidase activity:

Sections were incubated in 0.3 % H_2O_2 in methanol for 30 min. and washed with 0.05 M Tris buffer pH 7.6. Subsequent reagents were applied in drops directly over the sections. A circle was engraved around the sections on the glass slide with the help of a diamond pen to prevent the reagents from flowing away.

c) Sections were incubated with 3 % normal swine serum (code X 901 Dako, Denmark) diluted in 0.05 M Tris buffer, pH 7.6 for 30 min. at room temperature.

d) Excess of serum was blotted and sections were incubated with anti-Apis drone late larval, prepupal and pupal mixture serum (Primary antibody) diluted 1:200-1:800 in 1 % normal swine serum in Tris buffer for 72 hrs. at 4°C in damp closed petridishes.

e) Sections were equilibrated to room temperature during the last 1-2 hrs. and then washed in Tris buffer three times, changing it every 2 min. They were incubated with swine anti-rabbit IgG (link antibody, code Z 196 , Dako, Denmark), diluted to 1:15 in Tris buffer for 30 min. at room temperature.

f) After washing the sections in Tris buffer three times, changing every 2 min., they were incubated with Horseradish peroxidase-anti-peroxidase soluble-complex (code Z 196 resp. Z 113, Dako, Denmark) diluted 1:30-70 in Tris buffer containing 1 % normal swine serum for 30 min. at room temperature.

g) Sections were washed in Tris buffer as above and incubated at room temperature for 2-8 min. with 0.01 % H_2O_2 and 0.05 % N,N'-diaminobenzidine hydrochloride (DAB, code 18865, Serva) to develop the color reaction.

h) After stopping the reaction by washing the slide in Tris buffer,

they were dehydrated in alcohol series and mounted in Eukitt, observed and photographed with a Zeiss Photomicroscope. For specificity, either primary antiserum was omitted or replaced by normal rabbit serum. For control of the method, PAP, DAB, or immunoglobulins were omitted.

II. REPRODUCTION IN VARROA.

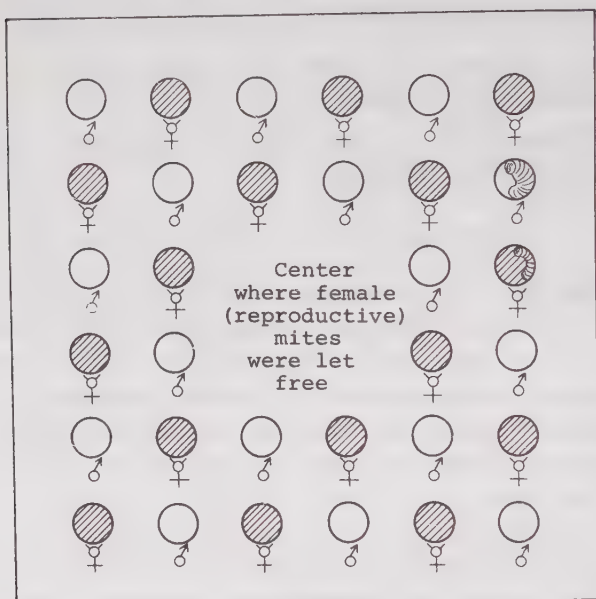
B.2.1. Preference of Apis drone larvae to worker larvae by Varroa female for reproduction.

B.2.1.1. Bio-test with Apis drone and worker larvae.

About 200 late larvae (equal number of drone and worker) were collected from very recently capped Apis brood cells from a colony free of Varroa infestation. Care was taken to collect drone and worker larvae with separate forceps to avoid any contamination. About 100 adult female mites were collected from the capped brood cells of an infested colony. A group of 10 mites were also collected from nurse stage bees.

Clean plastic trays (Multiwell disposotrays, Linbro, U.S.A.) punched with several parallel rows of pits were used for in-vitro bio-tests. At times, the trays were coated with molten bees' wax to imitate the natural combs. Alternately, drone and worker larvae were arranged in the pits each time using separate forceps. 7-30 reproductive female mites were freed in the middle of the trays with the help of a needle (sketch, next page). From the very beginning, the mites were observed for a few hours to register the course of locomotion. After different time intervals (5 min. to 36 hrs.) the mites sucking on either drone or worker larvae and those which moved elsewhere were counted and noted. This experiment was repeated 17 times, each time changing the trays and test animals. At times the drone and worker larvae were replaced with a few prepupae and pupae to check the host stage specificity. The pitted trays were kept in glass containers covered with transparent porous foils. This was in turn kept in the dark inside of an incubator at 34-37°C

with 70-90 % rel. humidity. The experiments were carried out during the breeding seasons of the bee as well as of the mites (May to September).



B.2.1.2. Kairomone test in-vitro and in natural bee colonies.

a) In-vitro

An *Apis* drone larval kairomone extracted with CH_2Cl_2 (Merck) and also a chromatographically separated fraction (prepared by courtesy of Prof. Howse, England) was used for this experiment.

In-vitro test was done in plastic trays already described in B.2.1.1. Small pieces of filter papers soaked in the kairomone solution were put alternately in the pits alone or at times with worker larvae. For one test, about 10 reproductive female *Varroa* mites (collected from late larvae, prepupae and adult nurse bees) were allowed to

move in the middle of the trays. In some experiments the filter papers (soaked) were replaced with drone and worker larvae and the test was repeated in the same way. The reaction of the mites was noted for short time periods (up to 6 hrs.) and the trays with the animals were kept in the incubator under the same conditions as described for B.2.1.1. for longer time intervals.

b) In natural bee colonies.

Rectangular, wooden boxes (18 x 3.5 x 1.3 cm) for trapping the mites were made with one side covered with a celluloid sheet. On both the sides, long slits of 2.5 mm were made through which only the mite could pass. The box was dipped in molted bees' wax to make it acceptable to Varroa as well as to the bees. Kairomone solution was applied to a 1 cm wide strip on the inner surface and adhesive tape or vaseline was added around it to trap the incoming mites. The boxes were suspended in the space between the combs (bee space) of the brood nest in Varroa infested colonies. Before hanging the boxes, the drone broods were removed and in some cases left as they were. It was expected that the reproductive mites, because of their attraction to the male bee larvae, would be trapped in the boxes. The experiment was repeated several times by changing the conditions and concentrations of kairomone solution (1:1-10,000).

B.2.2. Immunological and electrophoretical detection of Apis hemolymph proteins in the eggs of Varroa.

The eggs of Varroa were collected from the honey bee brood cells of the heavily infested colonies of Apis mellifera carnica. Capped drone and worker brood cells containing late larvae and prepupae were opened carefully under the stereomicroscope. The larvae were removed with the help of forceps, taking care not to puncture their body covering which might have resulted in contamination of the eggs of the mite. Usually 2-3 eggs, soft and whitish in color, are found on the walls of the brood cells. The eggs were collected and immediately frozen at -20°C. About 20-40 eggs of Varroa were homogenized with Barbitol buffer pH 8.6 and centrifuged

twice at 3,500 g. The supernatant was used for the immunological studies and in sample buffer (Tris-HCl, SDS pH 6.8) for SDS-polyacrylamide electrophoresis. The antigen amount in the egg sample was obviously lower than that of the hemolymph samples. Therefore, the amounts of egg sample available for the following experiments were less.

B.2.2.1. Ouchterlony-immunodiffusion in agarose gel.

The homogenates of the eggs (e) in the barbital buffer pH 8.6 were used for the immunodiffusion as already described under B.1.2.3. The arrangements of samples are illustrated in Fig. 6 (a) and (b).

B.2.2.2. Immuno-electrophoresis.

Preparation of agarose gel, running of gel, immunodiffusion and post diffusion treatment was similar to the method described in B.1.2.4. The only difference was in the running time which was in this case only 30 min. (fig.17). Three types of antigens were used, namely, Varroa egg homogenate (e), hemolymph from protonymph (p) and a mixture of drone late larval, prepupal and pupal hemolymph (D); Antiserum against the latter (AD) being the antibody for this test.

B.2.2.3. Two dimensional Tandem cross-immuno-electrophoresis.

The method used here was the same as in B.1.2.5. 5 µl of drone hemolymph (D) and Varroa egg homogenate (e) in Barbital buffer pH 8.6 were run for 45 min. in 1 % buffered agarose in the first dimension. In the second dimension 1.5 % anti-Apis worker prepupal serum (AA) (Fig.18) has been used. Running conditions and post-electrophoresis treatment were identical to that of B.1.2.5.

B.2.2.4. SDS-polyacrylamide slab gel electrophoresis.

3 % stacking gel and 6 % resolving gel were casted as described in the method of LAEMMLI (1970) under B.1.2.6. In this gel, the drone pupal hemolymph, Varroa female hemolymph and mite's egg homogenate in sample buffer were separated under the same conditions as before.

The electrophoretic separation of these proteins were further compared by scanning the gels (Fig. 19). Each band in the gel and the peak of the scanning pattern represents a protein. A peak in one sample identical to a peak of the another sample, illustrates the same migration rate, hence, identical proteins.

B.2.3. Immunohistochemical (PAP) localization of Apis hemolymph antigens in the vitellogenic oocytes of Varroa.

The reproductive female mites were collected by opening the freshly capped drone and worker larval cells. Few mites were collected from the nurse stage bees. Many mites had the eggs (ready to be laid) in the opisthosoma giving the pregnant females a swollen appearance. After removing their legs (for better penetration of fixative) they were fixed and embedded as described in B.1.6.2. Semithin sections (0.25-1.0 μm) were cut and treated in the same manner as in B.1.5.4. Whenever appropriate mites with medium sized oocytes were available, a few sections were cut and stained with Toluidine blue for light microscopy. The rest of the sections were stained by unlabeled-antibody-enzyme method to localize the honey bee hemolymph proteins in the vitellogenic oocytes.

B.2.4. Vitellogenesis in Varroa

B.2.4.1. Detection of Apis (host) and Varroa (parasite) hemolymph proteins in the hemolymph of Varroa by SDS-PAGE.

A comparison between SDS-PAGE electrophoretic patterns of Varroa female hemolymph and that of Apis developmental stages was carried out to differentiate the Varroa hemolymph proteins from resorbed Apis hemolymph proteins in Varroa's hemolymph. As already mentioned, the protein spectrum of all developing stages inside the brood cells is similar except the variations in the individual protein titres, which is closest to equilibrium in the pupal stage, the pupal hemolymph was selected for comparing the scanning patterns.

It was not possible to produce an antiserum against the Varroa hemolymph proteins because the mite contains a very small amount of

hemolymph, not enough to immunize a rabbit.

The method of SDS-PAGE according to LAEMMLI (1970) has already been described in section B.1.2.6. except that 4-5 % stacking gel and 10-15 % resolving or separating gels were used in this study for obtaining sharp bands. In small pore sized gels, the diffusion of protein sample is minimized. 10 μ l of Varroa and 5 μ l of hemolymph from different stages of honey bees were loaded in the wells for electrophoresis and run for 12 hrs. under similar conditons as before. The Coomassie blue stained gels were photographed and scanned with a 1:1 ratio.

B.2.4.2. Detection of foreign (Apis) and own (Varroa) proteins as vitellogenins of Varroa by SDS-PAGE.

The method of electrophoresis corresponds to that of section B.1.2.6. 5 % stacking gel and 12 % resolving gel were run under the same conditions. 10 μ l of hemolymph of Varroa and egg homogenate, 5 μ l of hemolymph from Apis pupae, prepupae and L₅ larval stage were used for this study. Electrophoresis was stopped when the tracking dye reached the end of the gel. The gel was stained with Coomassie blue, photographed and scanned as before. Scanning patterns of Varroa hemolymph and egg homogenate were compared in this run to identify the foreign and the mites' own vitellogenins.

B.2.4.3. Light and electron microscopic studies of the functional morphology of oogenesis in Varroa.

A. Varroa female during the winter diapause.

During the month of January, when honey bee colonies were totally broodless, about 20 mites were collected from the bodies of winter honey bees (Apis mellifera carnica).

B. Varroa females during the reproductive period in the summer.

About 150 Varroa female mites were collected during the months between April and August continuously for 3 seasons. The mites were gathered by opening the drone and worker brood cells containing late larvae, prepupae, pupae and from the bodies of emerging bees. A few

specimens were also collected from the bees in nurse stage to study the complete cycle of oocyte development.

Both summer and winter groups of mites were narcotized with CO_2 for fixation. The legs were carefully removed to facilitate the infiltration of fixatives. The mites were fixed and embedded in the same manner as described under B.1.6.1. The section cutting, post-treatment, staining and photography of semi- and ultra thin sections followed the procedure given in B.1.6.3.

To obtain the appropriate stages of oogenesis, a large number of mites were cut.

C. RESULTS

I. NUTRITION IN VARROA

C.1.1. Quantitation of the amount of Apis hemolymph volume sucked by the female mite, Varroa jacobsoni:

C.1.1.1. Volume of hemolymph in Apis drone and worker L₅ larvae determined by isotope dilution.

The concentration of injected ³H-inulin leveled off only in the drone experiment (Fig. 2b) and not in workers (Fig. 2a). Therefore, the calculated volume of hemolymph of about 53 μ l in drone L₅ larvae can be considered as representative, whereas the 44 μ l value for worker L₅ larvae is probably only a rough approximation.

As only a relatively small number of bee larvae were used in this isotope dilution experiment, in a scatter diagram the weight of the larvae demonstrated to fit within Gaussian's distribution (Fig. 2c & d). The body weight of the worker larvae used in these experiments ranged between 0.134 - 0.148 g and that of drone larvae was 0.348 - 0.399 g.

C.1.1.2. Honey bee hemolymph volume sucked by Varroa female under different conditions

The host hemolymph volume consumed by (in total 89) female mites under different conditions is listed in the table 1. In the first 45 min., no differences were found which could be related to the age of the female mites or the type of host (worker or drone larvae, respectively) and how long a hunger period had been. An increased feeding time was not significantly correlated with an increase in meal volume. Although such a tendency can be seen especially from the data obtained in the case of old and gravid female mites. Accordingly, the young mites sucked $0.86 (\pm 0.002)$ μ l upto 1.5 hrs without excrement, which were not deposited within this short period. An increasing tendency was found upto 3 hrs, during which the meal consumption

was $1.21 (\pm 0.002) - 1.30 (\pm 0.06) \mu\text{l}$. However keeping the mites without meal for 24 hrs had no apparent effect on the meal size of the mite.

The gravid mites with swollen opisthosoma which were collected from the late larval and prepupal stages consumed $0.83 (\pm 0.06) \mu\text{l}$ of host hemolymph within 1.5 hrs which was a lesser amount than the young ones. Perhaps the gravid mites consume the meal in little amounts but several times. Within 3 hrs there is an increase of $1.25 (\pm 0.01) - 1.26 (\pm 0.02) \mu\text{l}$. The mites collected from the pupal stage honey bees consume $1.27 (\pm 0.01) \mu\text{l}$ of hemolymph. The hunger period obviously did not effect a larger intake of blood. (Table 1., meal volume without excrements).

Upto within 6 hrs the young mites had sucked about $1.35 (\pm 0.02) \mu\text{l}$ of hemolymph and $1.50 (\pm 0.05) \mu\text{l}$ within 48 hrs including the excrements. The gravid female mite consumed $1.33 (\pm 0.03) \mu\text{l}$ in 6 hrs and $1.55 (\pm 0.01) \mu\text{l}$ hemolymph in 48 hrs. An increased consumption of meal was evident in the case of young as well as old mites relative to time taken. However, in case of old mites, there is more of a difference. A complete data over hemolymph volume of host consumed by Varroa is depicted in the scatter diagramm (Fig. 3) plotted with respect to volume against time in hours.

The following points must be taken into account during feeding the mites for a long period of time:

1. The mite may react against the artificial environment for prolonged period and act passively.
2. After long exposure the mite may start to react to the presence of a foreign molecule (^3H -inulin) in the host blood.
3. Since after approximately 48 hrs the labelled inulin would diffuse into the tissues and other body fluids of the bee larvae, the radioactivity present would not remain the same for a longer period of time.

The diagrams are plotted on the IBAS-1-Interactive Image Analysis System computer.

C.1.2. Immunological and electrophoretical detection of Apis hemolymph proteins in Varroa's hemolymph.

Following antisera against Apis hemolymph and its fractions were produced:

1. Anti drone serum (AD) against a mixture of equal amounts of late larval, prepupal and pupal hemolymph.
2. Anti worker prepupal serum (AA).
3. Anti worker hemolymph protein fraction H_1 (AH_1).
4. Anti vitellogenin (AV).

C.1.2.2. Radial immunodiffusion in agarose gel.

Fig. 4 represents the radial immunodiffusion. Centrifugal diffusion of hemolymph from drone pupae produces a series of concentric precipitin rings after 48 hrs of diffusion. Among them, 4 are distinctly visible. The hemolymph sample from female Varroa produced a similar ring formation. The number and dimensions of precipitin rings depend upon the concentration of antigens in the samples. The precipitin rings around the Varroa sample indicate the presence of antigens similar to the Apis hemolymph.

C.1.2.3. Ouchterlony double immunodiffusion.

The antigens present in the hemolymph of the mite react with anti drone as well as anti worker serum (Fig. 5). They both produce complete confluence in the three main precipitin bands. This indicates immunological identity of drone and worker pupal hemolymph indirectly. Among the three bands, one is also identical to an imaginal protein fraction H_1 (ENGELS, 1972) as represented by the line curving through AD. In contrast to this, no precipitin line is formed with anti vitellogenin (AV); as a matter of fact this protein is only synthesized in adult worker and queen bees. The Varroa hemolymph samples, however, were collected from those mites which fed only on larvae or pupae of the bee.

Further immunodiffusions show the presence of Apis hemolymph proteins in all the nymphal instars of the mite (Fig. 6). The hemolymphs from the proto- and deutonymph mixture, from the adult mite and Apis drone larval mixture, and from the prepupal and pupal hemolymph mixture all produce confluent precipitin bands with anti drone serum (Fig. 6 a) as well as with anti worker serum (Fig. 6 b). The spur formation (→) towards the Varroa antigen well and Apis hemolymph antigen well leads to the presumption that a slight proteolytic change occurs in the Apis hemolymph antigens during their passage through the mite's digestive tract.

C.1.2.4. Immunoelectrophoresis.

The immunoelectrophoretic patterns of drone late larval, prepupal and pupal hemolymph proteins by and large correspond to each other (Fig. 7). Precipitin patterns of female mite and the patterns of the mixture of proto- and deutonymph hemolymph are similar. All the precipitin arches of compared samples of honey bee and the mite are identical and confluent, except for the minute differences in shape and size, which could be explained as before, slight enzymatic changes during the process of resorption in the digestive tract of the mite.

C.1.2.5. Two dimensional Tandem-cross-immunoelectrophoresis.

In this technique two similar antigen samples can be compared both quantitatively as well as qualitatively. The formation of double peaks represents the identity of two samples and the integrals of the peaks area is proportional to the amount of antigen present in the sample.

In Fig. 8, the drone pupal hemolymph produces 7 peaks (the main fractions) in the anti serum (AD) impregnated gel. In Fig. 8 a, a few subsidiary fractions appear near the antigen wells (→ arrow).

Quantitative analysis was not possible, because of the individual differences in each fraction in different samples of Apis stages. Secondly the mixture of hemolymph samples from a large number of mites does not allow a standard antigen sample to be compared. Qualitatively

3 out of 7 peaks of the drone antigens also appear in Varroa female samples. The peaks areas are different. The fraction 3 in the drone and Varroa samples show a 2:1 ratio of peak integrals. In Varroa samples (Fig. 2.a) peaks 4 and 5 are underrepresented, and 4 of the Apis fractions (1, 2, 6 and 7) are not represented in Varroa samples. Four of the Apis fractions could be identified in the deutonymph samples. The ratio of the antigens in the drone sample is nearly identical, although the concentration of deutonymph sample is much lower as illustrated by the height of the peak.

By observing the Tandem patterns it becomes obvious that the Varroa female as well as its deutonymph stages resorb to different extents the host hemolymph proteins into their own hemolymph.

C. 1.2.6. SDS-Polyacrylamide slab gel electrophoresis (PAGE)

In Fig. 9 electrophoretic patterns of hemolymph of the Varroa female, Apis late larval, prepupal and pupal stages are compared in a single gel under the same conditions. The migration rates of almost all the bee proteins are identical to those of Varroa hemolymph, representing their identity and confirming the immunological finding of the previous experiments. The resolution of this technique is much higher than rest of the techniques used so far. In spite of the fact that all proteins of Apis pupae, prepupae and larvae are identical, their individual concentrations in the last two stages vary a great deal. For this reason gel scanning patterns of only Apis pupae and Varroa hemolymph were compared (Fig. 10). All the peaks of mite and bee samples correspond except numbers 1 to 5 in Varroa and A to F in Apis hemolymph. The absence of A to F Apis hemolymph proteins in the Varroa sample indicates their proteolytic degradation during their passage through the mite's digestive tract. Peaks 1 to 5 are Varroa's own hemolymph proteins. These findings correspond to the missing antigens in the Tandem cross-immunoelectrophoresis.

C.1.3. Ingestion of Apis drone and larval food by reproductive female Varroa

C.1.3.1. Radial immunodiffusion

As shown in Fig. 11, the drone larval jelly produces several concentric rings in the anti royal jelly impregnated gel. It indicates the identity of proteins in the two samples. However, around the Varroa homogenate samples there is relatively small precipitin ring formation, which indicates the presence of identical antigens in Varroa. The noticeable differences in the ring size is due to concentration of antigens. This indicates that the Varroa female ingests the larval food.

C.1.4. Undigested uptake of Bovine Serum Albumin (BSA) in the hemolymph of Varroa mite.

C.1.4.1. The results of immunodiffusions of hemolymph of Varroa females and that of honey bee, after injections of BSA (collected at 12, 24, 36, and 48 hrs) are given below.

C. 1.4.2. Radial immunodiffusion

As seen in Fig. 12, the BSA antigen appears in Apis and in Varroa hemolymph as precipitin discs. The quantitative comparison was not possible because the hemolymph samples of several mites were pooled to load one antigen well. However, it is qualitatively evident that Varroa resorbs not only the host proteins, but also the experimentally offered proteins in a similar manner.

C.1.4.3. Ouchterlony double immunodiffusion.

The production of confluent precipitin lines or bands makes more or less a hexagon (Fig. 13) around the antibody well without spur formation. This fact indicates a complete resorption of non-host protein (BSA) into the hemolymph of Varroa, thus confirming the results

of radial immunodiffusion. The total confluence of precipitation bands shows complete absence of peptide fragments of BSA in the hemolymph sample of Varroa. BSA could be detected as early as 12 hrs after feeding was initiated, and increasing amounts were found after longer periods of feeding. The concentration of albumin in the hemolymph of injected bees remained approximately unchanged up to 48 hrs. At the same time albumin concentration in the mite's hemolymph was found to be same as in the corresponding bees. This could be taken as an indication that there was almost no proteolytic change during the passage of BSA through the alimentary system of the mite.

C.1.5. Proteolytic enzyme assays of the midgut proteases of Varroa.

Following are the results obtained by various protease enzyme assays of Varroa midgut.

C.1.5.1. Photopaper test.

No visible white zones, under the drops of Varroa homogenate appeared on the treated photopapers even after prolonged incubation periods. In case of protease test mixture, distinct white zones of proteolytic breakdown of the gelatine film of photopapers appeared after the incubation.

C.1.5.2. Proteolytic activity in polyacrylamide.

In case of Varroa homogenate, no zones, either weakly colored or colorless, were observed against the dark blue background, when the electrophoretically separated proteins in the gel were incubated with BSA or hemoglobin. In the case of the separated protease test mixture and the midgut homogenate of Sacrophaga fly colorless distinct zones were visible. This indicates a very low or even absence of proteolytic activity in Varroa homogenate, at least detectable by this method, in contrast to the other two samples.

C.1.5.3. Overall protease activity with synthetic substrates.

A very low proteolytic activity in Varroa homogenate could, however, be detected by incubating it with the synthetic substrates such as Azocasein and Hide-Powder-Azure (Table 2.). The colour reactions were checked by comparing the extinction differences.

C.1.5.4. Specification of Varroa proteases.

The extinction differences for Varroa homogenate and the protease test mixture, after incubation with the synthetic substrates for endopeptidase (GPNA and BAPA), were checked. Varroa homogenate shows in contrast to protease test mixture, an extremely low endopeptidase activity, not sufficient to cleave the long polypeptide chains of Apis hemolymph proteins (see Table 2.).

C.1.5.5. Identification of Varroa exopeptidases.

In case of substrates for exopeptidases (Hippuryl-lysine and Hippuryl-phenylalanin) the Varroa homogenate, after incubation with them showed a fairly strong activity only with the substrate for carboxydase-A (Hippuryl-phenylalanin). This indicates that only carboxydase-A is present in the Varroa homogenate (Table 4.). During incubation with the substrate for aminopeptidase (L-Alanin-4-nitroanilid), the Varroa homogenate showed only about 10 - 30 % activity as opposed to 80 - 100 % activity of the test mixture, indicating the presence of aminopeptidase (exopeptidase) in very low quantities.

C.1.5.6. Protease inhibitor in Apis hemolymph.

The protease activity of the test mixture was reduced to 25 % when incubated with Apis hemolymph, as compared to 100 % normal activity. The inhibitory effect of bee hemolymph was observed by comparing the extinction differences in the samples with and without bee hemolymph.

C.1.6. Histological and immunohistochemical studies of the midgut and excretory tubes of Varroa.

A. Structure of midgut and ventriculus.

This is a large, flat, sac-like organ with its three pairs of lateral coecae occupying almost the whole dorsal and lateral sides of the body cavity. The median pair of coecae in Varroa are long and curved and extend up to the posterior part of the opisthosoma.

Histological examination of ventriculus coecae (Fig. 14) showed that a single layer of large columnar epithelial cells lie on a basement membrane. The cells of the midgut vary in form and size. Many are oval, round or pyramidal in shape. The cytoplasm contains vacuoles and a large nucleus. These cells are often projected into the ventriculus lumen and contain various types of spherules and granules. These proliferated cells are thought to help in digestion or collection of waste material (HUGHES, 1959). The brush border of the columnar epithelium was distinctly visible even under the light microscope.

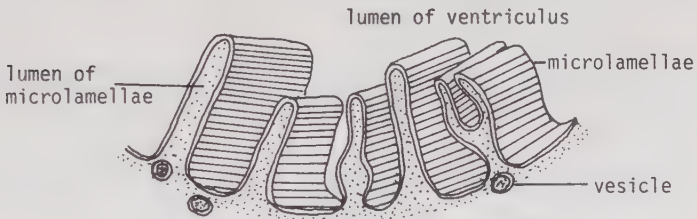
B. Fine structure of midgut.

The epithelium varies a great deal in thickness. It is bounded on its outer surface by a relatively thick membrane which appears homogeneous in composition. The outer surface of midgut coecum is highly fringed. The basal area of the epithelium is divided into many compartments by the infoldings of the plasma membrane (Fig. 15 c & d). The exocytotic pits (►) are often visible in this region (Fig. 15 c & d). Just outside the epithelium, adjacent to the basement membrane, are big spaces surrounded by plasma membrane and filled with electron dense material (Fig. 15 a).

The cytoplasm of epithelial cells contain relatively large nuclei. No Golgi zones or endoplasmic reticulum could be identified. The most abundant organelles are mitochondria. They range over a wide variety of size and shape. They are round, club shaped or dumbbell shaped with well developed cristae. Occasional collections of electron dense bodies are observed in the cytoplasm (Fig. 15 d & e). The vesicles were usually found empty, perhaps because the inclusions have

been washed away during fixation and post treatment.

The most unusual morphological feature of the midgut of Varroa is the lamellar border of the apical area of the epithelial cells, which posses bundles of ridgelike "microlamellae" (Fig. 15 d & e). At places they are situated in stacks and on a raised area. Some times they seem to be transitory between microvilli and microlamellae. Although the sections of this area were cut in all possible directions, no circular or elliptical profiles characteristic of the finger-like microvilli were observed. These ridges (microlamellae) are continuous with the long axis of midgut. The three dimensional reconstruction of the microlamellae is shown below:



The cytoplasm of the microlamellae does not contain to the same extent the organelles which are present in the epithelial cells: Some microlamellae appear to branch and others loop back and join the cell proper (Fig. 15 c). Between many of the microlamellae, small pits are present which appear to lead back to some distance into the cytoplasm.

The lumen of midgut ventriculum seems to be empty perhaps because the contents have been washed away during fixation and post treatment.

C. Structure of excretory tubes.

The epithelium of excretory tubes are uniform in thickness. The cells have large well defined nuclei. The apical border of epithelium is covered with fine microvilli.

D. Fine structure of excretory tubes.

The tubes do not show any particularly fine structure, the

lumen contains abundant bodies of round, spherical or rectangular shape. In the light microscopy these bodies shimmer and are made of Guanin, amin oxypurine (IONESCU-VARO and SUCIU, 1978). Towards the basal side of the epithelium, occasional invaginations or vesicle formation are seen. The cell to cell junctions in the epithelium are continuous with tight junctions or zonae occludens (Fig. 15 a & b). The cytoplasm contains relatively round and ovoid mitochondria and vesicles contain inclusions. Compartmentalization by plasma membrane towards the basal portion of the epithelial cells, as in the midgut, is absent in the excretory tubes.

C.1.6.4. The unlabelled antibody-enzyme method (PAP) for immunocytochemistry of the midgut.

Fig. 16 a shows the semithin cross section of female Varroa mite, stained with Toludin blue. A portion of the opisthosoma including the large distended midgut ventricle is visible. Sections from the same mite were treated for PAP staining as described under B.1.6.4. In Fig. 16 b the dark PAP shows the presence of Apis (host) originated antigens (Apis hemolymph proteins). The lumen is full with host hemolymph and the transport of honey bee macromolecular proteins through the epithelium into the hemocoelic cavity of the mite distinctly recognizable. This transport is seen as a gradient of stain reaching into the fat body surrounding the gut.

II. REPRODUCTION

C.2.1. Preference of Apis drone larvae to worker larvae by Varroa female for reproduction.

C.2.1.1. Bio-test with drone and worker larvae.

The observations of these experiments are depicted in Table 5. and summarized as follows:

- a. Even the blind reproductive female mite finds the host (bee larvae) within a short time of about 5 minutes.

b. The mites could very efficiently differentiate drone from worker larvae. Majority of the mites attached themselves to drone larvae. The percentage of mites attaching to the drone larvae was high in each experiment, sometimes amounting to 100 %, with the exception of the two experiments where only about 57 % of the mites behaved in this manner.

c. The bees wax coating over the plastic trays did not have any significant effect.

d. When drone larvae were replaced by drone prepupae and pupae, preference of the mite went to the worker larvae.

e. It was observed that the mites, while passing by a pit containing worker larvae, at times turned a little, raised and moved the first pair of legs (suggesting an olfactory reception of some type of stimulus) and then moved towards the drone larvae. There were, however, individual mites which went directly to drone larvae, ignoring on 2 - 3 pits containing worker larvae.

C.2.1.2. Kairomone-test in vitro and in natural bee colonies.

a. In-vitro observations:

1. The female mites which were left in the middle of the tray showed no particular interest after 2 - 5 min for the Kairomone soaked filter papers. Even for prolonged periods (6 - 24 hrs) no attraction for the drone extracts was observed.

2. In contrast to Bio-test, in the presence of Kairomone soaked filter papers, the majority of mites left the plastic tray and a suppressed attraction towards the drone and worker larvae was noted. It appears that the extracted Kairomone has a repellent effect rather than an attracting one.

b. In natural bee colonies.

The trapping boxes smeared with Kairomone solution with varying dilutions were checked for trapped Varroa mites. However, no mite could be found in these boxes. The results obtained here are in concordance to the previous in-vitro test.

C.2.2. Immunological and electrophoretical detection of host proteins in the eggs of Varroa.

The possibility of contamination of Varroa eggs from the hemolymph of Apis developmental stages was avoided by carefully removing the larvae and collecting the eggs from brood cell walls. The possible continuous streaking (leakage of proteins) during the electrophoresis of the egg homogenate sample was avoided by centrifuging the egg sample in order to remove the chorion and other embryonic membranes.

C.2.2.1. Ouchterlony immunodiffusion.

Studying once again Fig. 6 a and b one finds that the antigens in the egg homogenate (e) cross-react with anti Apis worker (nurse stage) serum (AA) as well as anti drone mixture serum (AD). 3 to 4 precipitin bands continue through the Varroa hemolymph containing wells (v), protonymph (p) and deutonymph (d). The same bands join with the hemolymph samples of worker nurse stage bee (A) and drone pupal hemolymph (D). However, between hemolymph of Varroa (v) and worker (A) in Fig. 6 b and drone pupal hemolymph (D) in Fig. 6 a, there are spur formations (→). Similarly, in Fig. 6 a, between drone pupal hemolymph (D) and egg homogenate of Varroa (e) there is spur formation (▶). It can be inferred that the honey bee hemolymph antigens are present in all the developing stages of the mite including the freshly laid eggs. But the minor changes in antigenic determinants (spur formation) are due to the low or suppressed proteolytic activity in the midgut of mite.

C.2.2.2. Immunoelectrophoresis.

In Fig. 17 the similarity in the precipitin arches formed by drone hemolymph (D), hemolymph of protonymph of Varroa (p) and egg homogenate (e) against the antidrone mixture serum shows the identity of the proteins. The full confluence and size of the arches is due to the short electrophoresis time (30 min).

C.2.2.3. Two dimensional Tandem-cross-immunoelectrophoresis.

The peak patterns of drone hemolymph (D) against antiworker serum (AA) and anti drone serum (AD) correspond with each other (compare Fig. 8 and Fig. 18). In Fig. 18 the 7 peaks of drone pupae are also distinguishable. In spite of the low concentration of egg homogenate of Varroa, 3 fractions (No. 3, 4 and 6) can be identified, which are continuing as double peaks with drone hemolymph sample. The concentration ratio of fraction 6 and 4 partially correspond. However, the fraction 3 in Varroa egg sample is much less. As mentioned before a quantitative analysis is not possible because with this method only the sample in question can be compared with the standard sample, whereas drone hemolymph sample contained a mixture of late larval, prepupal and pupal hemolymph, in which the individual protein titre varies in different stages.

C.2.2.4. SDS polyacrylamide electrophoresis.

Due to the big pore size of 6 % acrylamide gel the proteins in the sample migrate further from each other in comparison to 15 % gel. In Fig. 19 the protein separation patterns of drone pupal hemolymph, Varroa female hemolymph and egg homogenate of the mites are compared in the same run under identical conditions.

The gel scanning patterns of honey bee pupal hemolymph and Varroa egg homogenate are compared. About 20 - 25 protein peaks of egg sample superimpose the pupal protein peaks. This indicates their identity with each other, at the same time confirming the earlier immunological results. However, each pupal hemolymph protein present in the mites eggs does not correspond on the basis of their individual concentration to the proteins of pupal hemolymph sample. A group of proteins are taken up in high concentration, while the others are not.

C.2.3. Immunocytochemical localization of host (Apis) antigens in the vitellogenic oocytes of Varroa.

Fig. 20 shows a semithin crosssection of female reproductive Varroa, stained with Toludin blue. The section is passing through the middle of the body. The ovary (OV) is accompanied by two lateral lyrate organs (LY) (MICHAEL, 1892), typical structure in the members of Gamasidae. The function of these organs are still contradictory. On the posterio-dorsal side of the ovary, spermatheca (ST) is situated which is filled with spermatids in different developing stages. Adjacent to this and ovary there is spacious empty midgut ventricle (MV) surrounded by a thin wall. The ovary contains a number of oocytes in different stages of development. However, in all the appropriate sections studied, (20 - 25) only 5 - 7 oocytes (➡) were observed to attain the maturity and stay in medium size. Out of these oocytes one reaches double the size (vitellogenic). Rest of the oocytes remain very small. These histological observations confirm the earlier findings that Varroa mites lay 4 - 5 eggs in one brood cell singly at about 12 hrs intervals.

The medium sized oocytes are surrounded by a couple of membranes which make a pocket like cavity. In close contact to these oocytes are so called nutritive cells (N) which are degenerating oocytes and on this basis cannot be compared to insect trophocytes as often mentioned by many earlier workers. The largest oocyte possesses a big nucleus and 1 - 2 nucleoli. In light microscopy the cytoplasm looks undifferentiated and takes a uniform blue stain of Toludin blue.

Few sections of the same mite were treated for unlabelled antibody-enzyme reaction with the help of primary antiserum directed to Apis late larval, prepupal and pupal hemolymph mixture (Fig. 21). In the ovary only 5 medium sized oocytes are clearly labelled with PAP. Small oocytes, ovarian stroma, and surrounding tissue, such as subepidermal fat body, spermatheca and lyrate organ remained unstained. Results like this were obtained from all female mites in appropriate stages of development or oogenesis. In control sections no PAP labelling could be seen.

The sensitivity and specificity of this technique was controlled by observing the surrounding tissue of the darkly stained oocytes and the controls, where there was absolutely no contrast between the oocytes and the surrounding tissue. Fig. 22 shows the enlargement of the same section. In the enlargement the contrast is more evident, leaving the nucleus unstained.

C.2.4. Vitellogenesis in Varroa.

C.2.4.1. Detection of Apis and Varroa hemolymph proteins in Varroa's hemolymph.

Fig. 23 a shows the comparison between the electrophoretic patterns of the hemolymph proteins of Varroa and Apis (host) pupal, prepupal and late larval stages in SDS-PAGE slab gel under identical conditions. The migration rates of the proteins were different due to the difference in the pore size of the gel. In Fig. 23 a and b the protein bands are closer and sharper in the 15 % separating gel as compared to the gel of 10 % concentration (Fig. 23 c).

The proteins Nos. 1 - 5 were identified as Varroa's own hemolymph proteins, whereas proteins A - F belong to that group of honey bee hemolymph proteins which are absent from the hemolymph of the mite. By comparing the scanning patterns of Apis pupal and Varroa female hemolymph six (A - F) honey bee proteins could be detected that have no counterpart in Varroa's hemolymph. With the help of this method of electrophoresis each hemolymph sample could be separated into 30 - 50 or more protein bands. Some of these migrate very close and some that are present in high concentrations resulted in superimposition on other minor proteins.

C.2.4.2. Identification of foreign (Apis) and Varroa's own hemolymph proteins as its vitellogenins by SDS-PAGE.

Fig. 24 a shows a pattern of 12 % polyacrylamide gel and 24 b a comparison of the scanning patterns of Varroa egg homogenate and its hemolymph, allowed to run in the same gel. A comparison of elec-

trophoretic patterns of hemolymph of Varroa and its egg homogenate and hemolymph of Apis pupae, prepupae and late larvae showed that 20 - 30 proteins in the same run had identical migration rates (Fig. 24 a). Thus it was evident that Varroa mite resorbs host proteins without significant digestion into its own hemolymph. Many of these resorbed proteins are later taken up by the developing oocytes; these have been identified by electrophoretic patterns of egg homogenate.

It was interesting to find that proteins Nos. 1 - 8 (host originated, except No. 2) were in higher concentrations in Varroa's eggs than in its hemolymph; in contrast to the resorbed host hemolymph proteins A - I that are in a higher concentration in Varroa's hemolymph. These findings enable these proteins Nos. 1 - 8 to be classified as vitellogenins of Varroa. Differential accumulation of host proteins by Varroa's eggs becomes evident by comparing proteins Nos. 1, 3, 4, B and C in the eggs, hemolymph of the mite and that of the host (Fig. 24 a). The 2 main Apis hemolymph proteins (B and C) which are present in high concentration in all the developing stages of Apis are accumulated in very low concentrations in Varroa's eggs as compared to its hemolymph.

C.2.4.3. Light and electron microscopical studies of the functional morphology of oogenesis in Varroa.

The club shaped ovary is situated almost in the middle of the opisthosoma together with a pair of short lyrate organs. The short oviduct leads into inconspicuous uterus which in turn opens into the chitinized vagina, whose opening is situated under the genitoventral plate. The ovary is surrounded by a thin layer of endothelial cells. During the reproductive period when female mite is active in egg-laying the ovary occupies the area extending from dorsal to the ventral side of the body cavity.

Adjacent to the ovary towards the dorsoposterior side there is a pouch like spermatheca, often filled with spermatophores in various developmental stages. A pair of tubuli annulati (MICHAEL, 1892) is also present in Varroa.

The germinal part (Fig. 25 and 26) of the ovary is centrally situated. The more developed oocytes are found near the periphery in sac like spaces which are lined with membranes. These sacs are not comparable to the typical outpushings of the ovary of Arachnids. Interspersed between the developing oocytes there are few interstitial cells. The degenerating oocytes or the nutritive cells are also present; these are characterized by distinct nuclei in syncytial mass just under the oocytes.

On the basis of electron microscopical observations, the oocytes of Varroa can be differentiated into 4 classes:

1. Degenerating oocytes (nutritive cells)
2. Functional oocytes
3. Previtellogenic oocytes
4. Vitellogenic oocytes

1. Degenerating oocytes (nutritive cells).

These cell masses are often called as nutritive stroma (REUTER, 1907; IONESCU-VARO and SUCIU, 1979). Small oogonia present in the close vicinity of the functional oocytes, show a karyolytic degeneration and never become functional. The cell inclusions of these oocytes seem to help in the growth process of developing oocytes and this phenomena is similar to the observations made by PLUMPH, 1954 in honey bee mite, Acaripis woodi.

In Varroa there are extra ovarian cells found singly (Fig. 29) or in groups, lying adjacent to the ovary between spermatheca and the ovary (Fig. 26). These cells lie freely in the hemocoel. They resemble the group of cells described by PAHNKA, 1974 in female Limnochares aquatica near the alimentary canal and ovary and were thought to function as protein synthesizing organs during egg development. THOMAE, 1926, reported similar type of cell clusters in the females of Halacaridens and in Allothrombium, HENKING, 1882, named them "fat bodies". In Bdelliden, ALBERTI, 1974, renamed them as "lateral cells". PAHNKE and WITTE, 1974, conclusively gave them the term extra ovarian nutritive cells, because of their differential function in Trombidiformes group.

2. Functional oocytes.

All other oocytes situated in the germinal part of the ovary not showing any type of karyolytic degradation are functional oocytes. They acquire the capacity to undergo vitellogenesis when required. They are 5 - 20 in number and usually oval in shape, possessing a large nucleus with 1 or 2 nucleoli. The cytoplasm of these oocytes stains dark with Toludin blue. They have well defined oolemma.

The above two types of oocytes are found in the mites of winter reproductive diapause and summer early reproductive phase as well. The next two types of oocytes were encountered in the mites during the summer reproductive period. They are the previtellogenic and vitellogenic oocytes and will be described later in section B. However, the winter reproductive diapause oocytes can be included in the previtellogenic oocytes of first order, while, except for little differences, they resemble the summer oocytes.

A. Functional morphology of oocytes during winter reproductive diapause.

Fig. 25 shows the structure of ovary during winter (January, February) in Varroa females who over-winter by attaching themselves onto winter bees. About 10 - 13 small round and oval oocytes are seen which have more or less the same size in contrast to the summer oocytes among which 2 - 3 oocytes always go into previtellogenic phase and attain more than double the size of the other sister oocytes.

Under the electron microscope (Fig. 27) the winter diapause oocytes show a very unusual characteristic. All the oocytes, from smallest to the biggest, are fully packed with well demarcated mitochondria. This remarkable fact has never been described in other groups of mites. The mitochondria are often very long and branching (Fig. 27 b). Some transitional stages were observed in a few oocytes. In 1 or 2 oocytes the mitochondrial cristae seem to be disappearing and at the same time membrane bound vesicles arising with electron dense material inside the mitochondrial lumen (Fig. 27 c). The oocytes are surrounded by well demarcated oolemma. Just under this there are golgi zones with smooth parallel membranous cisternae and vesicles. The golgi bodies

are also scattered throughout the cytoplasm. In the oocytes where mitochondria start producing the vesicles the golgi zones also sometimes show vesicle formation (Fig. 27 d). The rough endoplasmic reticulum and the cisternae appear to be associated with vesicles as in the case of mitochondria and golgi bodies. The functional participation of all these cellular organelles in the oogenesis by associating them with vesicles seems to resemble the type described by MOTHES and SEITZ, 1980, in the previtellogenic oocytes of Cupiennius salei and Tegenaria derhami.

Each oocyte is surrounded loosely by a couple of membranes other than the oolemma. In the interoocytal spaces there are groups of comparatively small mitochondria without distinct cristae. The nuclei of endothelial cells are also to be seen in these spaces (Fig. 27 a).

The nuclei of the oocytes are big with condensed and at times vacuolated chromatin material. The dense round nucleoli lie close to it. These oocytes which are at an arrested stage of development are filled with mitochondria; this was observed in all mites (ca.10) collected during winter for this study.

B. Functional morphology of oocytes during summer reproductive phase.

1. Light microscopy:

Usually the mites collected in summer breeding season of host and parasite (Fig. 24 and 30) contained 5 - 8 oocytes in their ovaries. However, occasional specimens contained the same number of oocytes (5 - 20) as in winter diapause (Fig. 28). During this active phase because of the growing oocytes the topography of the ovary changes and it occupies a larger area of the body cavity.

Varroa female lays one egg at a time (SMIRNOW, 1979). As there is no gonotrophic harmony, the Varroa female sucks the hemolymph of the host and the eggs become mature. This phenomena can be confirmed by observing the ovary of the mite in the summer breeding season. Only one vitellogenic oocyte attains 10 - 20 times larger volume than the rest of the oocytes. 2 - 3 oocytes resemble winter diapause oocytes in possessing almost similar cell organelles (compare Fig. 25 to

28 and 29). The exception being the decrease in the number of mitochondria. The cytoplasm takes a stronger stain with Toludin blue because it starts some yolk deposition (Fig. 27 b & 32). They are the "oocytes of first order" of previtellogenesis. The nuclei also show much diffused chromatin material as compared to the condensed type in winter diapausing oocytes (compare Fig. 27 b with 28 b & 32).

Two to three oocytes in summer become 3 - 5 times bigger than the others. They can be designated as "oocytes of the second order" of previtellogenesis (Fig. 28). The cytoplasm of vitellogenic oocytes (Fig. 30 b and 34) contains a few degenerating mitochondria which are being replaced by membrane bound vacuoles. The vitellogenesis in Varroa seems to differ from that described for other mites. The egg in Varroa mite is filled with big yolk spheres (Fig. 30 a).

2. Electron microscopy:

The previtellogenic oocytes are surrounded by a rather thick oolemma (Fig. 31 a and 32) which at places show occasional foldings. The golgi zones visible just under it show many dictyosomal cisternae. The cytoplasm contains free rough endoplasmic reticulum and ribosomes and is filled with mitochondria of various shapes and sizes. The lamellae of these mitochondria appear to be reduced in number and size in advanced previtellogenic stage oocytes and they have heavy matrix containig developing mitochondria described by MOTHES and SEITZ, 1980, in Tegenaria derhami. Mitochondria are integral (or indispensable) cell organelles of Varroa oocytes. They are present in all the phases of developing oocytes. Even during active vitellogenesis they are present centrally in the oocytes.

Nutritive cells or degenerating oocytes:

A great number of oogonia adjacent to the functional oocytes in the ovary during vitellogenesis undergo karyolysis and degeration. During the development of a single vitellogenic oocyte a great number of oogonia undergo degeneration to help this oocyte to develop within a short time. This indicates the "alimentary egg development". REUTER, 1907, described these abortive and fusing oogonia as "trophocytes" (Fig. 31 a & b). In Varroa the part of the ovary which contains

such oogonia was named "nutritive stroma" in the preliminary studies of IONESCU-VARO and SUCIU, 1979. Fig 31 shows two types of oogonia near the margin of previtellogenic oocytes. The oocyte with well defined nucleus and nucleolus was once the small functional oogonia, with well defined mitochondria and other cytoplasmic inclusions. The other oogonia have already entered into the process of irreversible karyolytic degeneration. Such degenerating oogonia are seen in Fig. 31 b. where the nuclei of their cells show pseudopodia like structures. The fragmented chromatin material is much diffused and appears to pass out through the pores in nuclear membrane; several such nuclei without cell boundaries were found. The cell inclusions are free to move into the interstitial space between the developing oocytes. The cytoplasm is finely granulated and possesses a few poorly functional mitochondria.

Previtellogenesis:

The characteristic feature of early previtellogenic oocytes is the absence of cytoplasmic yolk droplets. The cytoplasm is filled with numerous ribosomes, polysomes, vesicles and a large number of mitochondria which show a process of transformation and contain vesicles and electron dense material. There is an increase in the number of golgi bodies and multivesicular bodies (Fig. 31 a).

The late mitochondria of the previtellogenic oocytes (Fig. 32 b) show degeneration and are replaced by big vacuoles. The nucleus shows few electron dense nucleoli and chromatin material diffusing out into the cytoplasm through the highly porous nuclear membrane. Densely stained material is seen in juxtaposition to the entire periphery of the nucleus (Fig. 32). The smoothness of oolemma no longer exists.

Vitellogenesis:

The beginning of vitellogenesis in Varroa is landmarked by the degeneration or transformation of mitochondria and the specialization of oolemma. The endogenous yolk formation during the latter part of previtellogenesis is coupled with exogenous yolk uptake. In the peripheral cytoplasm mitochondria either almost disappear or start disappearing from one side of the oocytes (Fig. 34 b). The yolk spheres

appear among the endoplasmic reticulum. The morphology of the cell organelles in 2 - 3 medium sized oocytes starts changing. The mitochondrial lamellae during this stage look parallel to the long axis of the mitochondria (Fig. 33 a).

The cytoplasm contains multivesicular bodies and vesicles. The oolemma throws folds into the perivitellin space, which appear to loop back and at times contain electron dense material. A peculiar passage mechanism of material (yolk) from the surroundings in to the cortical region of vitellogenic oocytes in Varroa seems to be atypical.

An unusual type of microlamellar brush border only on one side of the vitellogenic oocytes was observed in a very small number (4 %) of mites (Fig. 33 b). This is unlike the typical type of microvillar brush border in other vertebrate or invertebrate animals. These structures which can be named as "microlamellae" are thick and branched enfolding the oolemma and are filled with electron dense material, which is observed when they were cut transversely (Fig. 33 b). Also in Varroa, near the oocytes, there are always a group of haemocytes which function as mediators between hemolymph and oocytes for the yolk uptake as suggested by SEITZ, 1974, in Cupiennius salei (Aranae, Ctenidae). Under the microlamellae the cortical ooplasm contains many yolk spheres, multivesicular bodies, degenerating mitochondria and free ribosomes.

Fig. 35 also shows small to big yolk spheres and coated vesicles which are the cytological manifestations of protein uptake by the vitellogenic oocytes from the hemolymph (ROTH and PORTER, 1964).

The chorion or egg shell formation starts in the oocytes, after it reaches the full size. This seems to be similar to that of Tetranychus urticae (Acari, Tetranychidae) described by MOTHES and SEITZ, 1981. The material for egg shell formation is endogenous in origin (occurs in the absence of follicular cells, like in insects) and is synthesized by oocyte itself. In the cortical ooplasm there appear several vesicles with dense, finely granular material. They appear to migrate towards the periphery, coalesce and form the egg shell by emptying their inclusions (Fig. 35 a & b). These vesicles are easily distinguished from the coated vesicles or yolk vesicles in having less electron opaque material.

D. DISCUSSION

I. NUTRITION IN VARROA.

D.1.1. The physiology of nutrition in Varroa.

Varroa jacobsoni is an ectoparasitic, stenophagous mite. In the real sense it can be called a "parasite" of Apis cerana, where it has never been reported to have caused the death of the colonies. However, in Apis mellifera, the mite is in fact "parasitoid", killing the bee colonies in case of heavy infestations. The mite sucks hemolymph from all the stages of honey bees except the 1 day to 4 days larvae (L_1 - L_4 larvae). The nymphal stages of Varroa also suck the blood of corresponding developmental stages of the bee in the sealed brood cells. Hence, honey bee hemolymph remains the only food available for nymphal instars as well as the adult mite.

In hemophagous mite of related family Gamasidae, the female swells after the blood meal, during occasional contacts with the host (SMIRNOW, 1978). However, in Varroa this phenomenon could not be observed perhaps, due to the steady availability of the host.

Before egg laying, the reproductive mite feeds on the hemolymph of the larval stages of the bee (1 week unsealed brood). The eggs are layed in the prepupal stage of bee, which corresponds to the reduction of juvenile hormone titre in the hemolymph of the bee stage (SMIRNOW, 1978).

Hence it is interesting to know the total hemolymph volumes of male and female larvae of the bees, which are determined with the help of "isotope dilution method" in this study. It amounted to 44.0 - 44.39 and 52.2 - 54.6 μ l respectively. This difference in the hemolymph volume could be correlated to the successful reproduction in male brood cells, perhaps where it does not disturb much the physiological status of the host larvae.

The precise data for the blood meal volume consumed by female mite (young, adult, reproductive, etc.) at different time intervals is not available. SMIRNOW, 1978, weighed hungry and fed Varroa females which had sucked on the worker bees for 2 hrs and recorded an increase

in the body weight of the mites up to 0.08 - 0.14 mg which was about 26 - 41 % of the mite's body weight. MORITZ, 1980, fed ^{32}P containing syrup to bees and allowed mites to suck. After 24 hrs he measured the radioactivity in the bees and in the mites. According to his calculations a young and old mite takes about 0.25 μl of host hemolymph.

These vast differences in the calculation of meal volume seems to be partially due to the different methods used by these 2 workers. However, a comprehensive description of the developmental and physiological status of host and parasite has been overlooked. After considering all these parameters, with the help of " ^3H -inulin dilution method", the following results were obtained:

- Keeping the mites hungry even for 24 hrs had no effect on the meal size of young or old mites. This is in agreement with the findings of MORITZ, 1980. However, the amount of meal volume is much below what was given by SMIRNOW, 1978, and calculated in the present study. This fact indicates that the mite can only consume a limited volume of host hemolymph and at a particular rhythm.
- Within a short time of 1.5 hrs both young and adult mites take almost equal amount of hemolymph from the late larvae of drone and worker bees. However, with the increase in time from 6 - 48 hrs the volume also increases in both the cases, only in the old mites the difference is little more.
- There were no discrepancies in the meal size, when the mite sucks on adult nursing stage (8 days old) bees.

The above findings point out to the fact that Varroa mite possessing hard cuticular covering can possibly retain a definite volume of host hemolymph. Even the adult reproductive mite takes the same amount, independent of hunger periods. On this basis it can be speculated that perhaps a definite volume of meal is important to maintain a certain osmotic pressure for locomotion and resorption of nutriment in the alimentary system of the mite.

Such data for the estimation of hazards to bees and for any systemic control method (in which the chemotherapeutic agents are to be introduced to the mites through the host hemolymph) as the assessment of the meal volume and frequency of sucking are of great impor-

-tance.

The immunological analysis of hemolymph of Varroa female mite in agarose gel with the antibodies directed against honey bee hemolymph, shows that the mite resorbs almost all the blood proteins of host without any significant degradation. Although the seldom appearance of spur formations in Ouchterlony immunodiffusion patterns shows a very small change of a few antigenic determinents. Not only adult mites but also their proto- and deutonymphs resorb the host proteins in the same manner. The Varroa hemolymph contains no peptide fragments of Apis proteins, but, instead contains intact proteins with partially modified antigenic characteristics.

In the present study only immune-tests were carried out with antibodies against Apis hemolymph proteins. Therefore, it was not possible to evaluate the other constituents of nutrition such as cell proteins, substances like sugars, amino acids etc. dissolved in bee hemolymph.

The SDS-polyacrylamide electrophoretic patterns of Varroa female and bee hemolymph confirm the immunological findings. The better resolution of this method allows almost all the hemolymph proteins to be compared. The few spur formations in Ouchterlony and missing proteins in Tandem patterns can be confirmed in this sensitive electrophoretic technique, in which about 6 Apis hemolymph proteins do not appear in hemolymph of Varroa. This suggest a minor proteolytic degradation of these proteins during the passage through the midgut of the mite. On the other hand, with the same method about 5 mite's own hemolymph proteins could be differentiated.

The findings of the above mentioned studies can be used in the control of Varroaosis in two ways. Firstly, the immunotests and electrophoresis of Apis proteins in the mite's hemolymph offer a possibility of predicting quantitatively, whether the mite has consumed a normal meal. This could be used for developing a sensitive test for a physiological effect of varroastatic acaricides. Secondly, the data for hemolymph ingestion by the mite are important to know. In the case of systemic agents which affect the mite through the hemolymph of the bees, the dose (MORITZ, 1980; RADEMACHER, 1980; RUTTNER, 1980; RUTTNER et al., 1980 b; TOSCHKOFF, 1980), resorption and effect of such subs-

-tances are important to know.

DE JONG, 1982, in South America observed in africanized bees that the female reproductive mites enter an open cell with late larva and initially feed on it, crawl under the larva and immerse themselves in the larval food until the larva finishes the food and cleans the cell before spinning the cocoon. However, it was not possible to state definitely whether the mite ingests the larval food.

With the help of immunotechnique, using antiserum against the larval food, it was observed that the mite indeed ingests the larval food, however, in small quantities. For the first time it was observed that in Apis mellifera carnica colonies also the mite immerses itself in the larval food before beginning the reproductive cycle. Such mites were also found in 3 - 4 days old (L_4 stages) larval cells in their food. According to SMIRNOW, 1978, the egg laying starts when the female mite has sucked on 1 week old unsealed larvae. It is repeatedly mentioned in the literature, that female mite enters the cell just before capping. But as per this study it seems that the mite enters open brood cells a little earlier. It can be speculated that the larval food plays some role, at least in initiating vitellogenesis in the mite.

There is clear evidence of macromolecular protein resorption by Varroa mites into their own hemolymph without significant digestive degradation of host (Apis) proteins. Not only host hemolymph proteins but also experimentally introduced vertebrate protein like bovine serum albumin (BSA) was resorbed into the hemolymph of the mite in the same manner. This was confirmed by immunetests. Macromolecular resorption of host proteins is also known to occur in few other Arthropod species (NOGGE, 1980; WIGGLESWORTH, 1943). Recently an intracellular passage of blood meal proteins in a mosquito was suggested by HOUK and HARDY, 1979.

The undegraded resorption of Apis hemolymph proteins and BSA raised the query what type of enzyme equipment (especially protease enzymes) is present in the midgut of Varroa. By evaluating the results of enzyme assays of Varroa alimentary system, the presumption that only a low proteolytic activity is present in the mite, was confirmed. Although for technical reasons the whole body homogenates of mites was

used for this study, it is suggested that the small quantities of proteolytic enzymes belong the midgut. The cellular lysosomic protease would not show activity under the pH regimes used here. Small amounts of exocarboxypeptidase-A type and aminopeptidase and traces of endocarboxypeptidase, with the help of very sensitive synthetic substrates, were detected in Varroa. Such an insufficient enzyme equipment of the mite's gut and effect of experimentally detected inhibitory factor of Apis hemolymph on protease activity can effect only a slight reduction in the molecular weight of the ingested proteins and not a complete breakdown of the polypeptides into small oligopeptides.

D. 1.2. Functional morphology of midgut and excretory tubes and immunocytochemistry of midgut.

A macromolecular host and non host (BSA) protein resorption by Varroa through its alimentary system made the study of functional morphology of midgut and excretory tubes necessary. The fine structure of midgut epithelium, which suggests an increased area of resorption, and the presence of exocytotic pits indicate the transport of resorbed nutrients into the hemolymph of the mite.

The most unusual and unique feature of the Varroa midgut ventriculus is the presence of "lamellar border" as opposed the usual striated or brush border of the apical area of midgut epithelium. Such specialized ridge-like structures have been described in very few other mites or insects. A few examples are in the midgut of Australian sheep blowfly larvae, Lucilia cuprina (WATERHAUSE and WRIGHT, 1960), in the malpighian tubules and hindgut of Macrosteles fescifrons (SMITH and LITTAN, 1960), and in the excretory tubes of the mite, Macrocheles muscae domestica (COON and AXTELL, 1967). The participation of such specialized epithelial border of midgut in macromolecular resorption of host proteins by Varroa during this study could not exactly be correlated, because no cytochemical studies in this direction were available. However, at least the looping back of microlamellae and occurrence of endocytotic pits in between them indicate some transport of nutrients from midgut lumen to the hemocoel.

The excretory tubes of Varroa do not show any significant structure histologically. The lumen is filled with multishaped bodies made of Guanine (amin oxypurine) which is typical of most of the arachnids (IONESCU-VARO and SUCIU, 1978).

The staining by unlabelled-antibody enzyme method (PAP) of the semithin cross sections of Varroa shows dark staining of the antigens (Apis hemolymph proteins). Through immunocytochemical staining (PAP) it is confirmed that Varroa mite resorbs the host proteins without degradation through midgut into the hemolymph, which bathes all organs including the ovary. Why the mite has developed such a mechanism for the ingested nutrition will be discussed in the following section, where physiology of reproduction in Varroa is being discussed.

II. REPRODUCTION. IN VARROA.

D.2.1. Parasitic adaptations of Varroa for reproduction.

KOENIGER et al., 1981, observed in Apis cerana (original host) that reproductive female Varroa enters both worker and drone brood cells, but lays eggs only in the latter. On the other hand, in Apis mellifera the mite prefers the drone cells to the worker cells and lays eggs in both (GROBOV, 1977; RUTTNER et al., 1981). These workers observed this behaviour of the mite while checking the sealed brood cells for their number. The results of the in-vitro tests of the present study are in agreement with that of the above tests. Varroa mite without any hindrance orients itself more to the male larvae than female. Development and results of such tests are of great values in the biological control (pheromones, kairomones, hormones etc.) of the mite.

The fate of host hemolymph proteins followed by immunotechniques and electrophoresis resulted in detecting the host antigens in the mite's own hemolymph. To follow the further course of these molecules from the hemolymph of the mite, the most interesting results were obtained. The immunological and electrophoretical detection of Apis proteins with the help of different techniques in the freshly laid Varroa eggs opened a new insight in the reproduction of Varroa jacob-

-soni. The minor spur formations (change in antigenic determinants) in Ouchterlony patterns was supported by electrophoretic patterns of Apis and the mite's egg homogenate, also where few host proteins present in the mite's hemolymph are not represented in the eggs. At the same time a group of proteins are accumulated in the eggs against the concentration gradient while others are not. This process can only commence during oogenesis and the proteins are in fact taken from the mite's hemolymph. Such examples of detecting foreign proteins in the ovary or eggs are very rare (WIGGLESWORTH, 1943; NOGGE, 1980). However, the significance of such mechanisms was not established in those studies.

The presence of host proteins in the eggs through these studies in Varroa, naturally called for a histochemical investigation of developing oocytes in the mite. By immunocytochemical (PAP) technique, the Apis proteins (resorbed from the blood meal) were distinctly localized in the ooplasm of growing oocytes of Varroa. As small oocytes are apparently free of honey bee proteins, the incorporation of macromolecular host material is restricted to special developmental period stages. Fully grown oocytes and embryos were also found densely packed with Apis proteins. However, the light microscopic resolution of the semithin sections did not with certainty establish histologically, whether the Apis hemolymph proteins were euplasmic or deutoplasmic components of the ooplasm of Varroa oocytes. Nevertheless, frequently the fully grown oocytes and embryos were observed to contain yolk spheres filled with host proteins.

The results of this immunohistochemical studies confirm the earlier studies, that Apis hemolymph proteins are resorbed by the mite undigested and are present also in Varroa eggs.

D. 2.2. Vitellogenesis in Varroa.

With the help of SDS-PAGE and after comparing their scanning patterns, five of the mite's own hemolymph proteins were identified and about six Apis hemolymph proteins were found to be missing in the mite's hemolymph. It corresponds to the earlier studies of enzyme assays of Varroa midgut proteases. The selective resorption of host

proteins indicates a high degree of parasitic adaptation in the mite.

On the other side, it was interesting to find out, that 7 host hemolymph proteins are accumulated in the Varroa eggs in higher concentrations than in its hemolymph. At the same time, the two most concentrated proteins of the host were detected in lower concentrations in the eggs than in the hemolymph of the mite, in contrast to above. Such selective uptake of hemolymph proteins by Varroa eggs against the concentration gradient enabled them (7 host and 1 own proteins) to be classified as vitellogenins of the mite. The criteria for calling proteins vitellogenins in the hemolymph of the female were suggested by HAGEDORN and KUNKEL, 1979, as follows:

1. Vitellogenins make 60 - 90 % of the soluble yolk proteins of the eggs.
2. They are not synthesized by oocytes themselves, but are synthesized in fat bodies or surrounding tissue.
3. They are present in high concentration only in females, hence are called female specific proteins.
4. They are selectively taken up by oocytes during the vitellogenesis, so that they are 20 - 100 times more concentrated in the oocytes than in the hemolymph.

The second and third criteria do not correspond to Varroa vitellogenins fully. Although a single own hemolymph protein of Varroa was found to be accumulated in the eggs, the most significant process in Varroa oogenesis is the uptake of foreign proteins (Apis hemolymph proteins) as vitellogenins.

In chelicerata very few studies have been done on yolk synthesis or vitellogenesis. DUMONT and ANDERSON, 1967, in Limulus observed that during early vitellogenesis, endogenous yolk granules are formed near the golgi bodies. In the main phase of vitellogenesis, the exogenous yolk is incorporated in the oocytes through micropinocytosis. A similar process was reported for ticks by JENNI, 1971, in Dermacentor andersoni by BRINTON and OLIVER, 1971. In spider the vitellogenesis is more complex (NATH et al., 1950; OSAKI, 1971). MOTHES and SEITZ, 1980, in Cupiennius salei Keys and Tegenaria derhami (Aranae) observed that the participation of endogenous and exogenous factors play a part in the yolk synthesis. During the endogenous phase the mitochon-

-dria, other organelles like golgi bodies, ER have been found to be associated in the yolk synthesis. During previtellogenesis in arachnids and insects with panoistic ovarioles, oocyte mitochondria may be required to play an important role relative to proliferation, morphogenesis, protein yolk formation, especially because follicle and nurse cells are nearly or completely absent.

A similar type of endogenous activity of the ooplasmic organelles was observed in the oocytes of Varroa during the previtellogenesis. However, for technical reasons, high power electron microscopy could not be done for each of these organelles.

During the winter reproductive diapause in the Apis mellifera colonies the oocytes of Varroa show a peculiar characteristic, in having their ooplasm fully packed with well-defined, functional mitochondria. The density of these organelles was found to be so high that it allowed almost no space for the others. This might be true of Apis mellifera colonies in suboceanic and subcontinental climate of Europe, where during winter, no Apis brood is available for mites egg laying. Such a phenomenon is perhaps an adaptation in Varroa to keep the oocyte active during this unfavourable condition, so that, when the first bee brood appears, the mite can readily start oogenesis, as soon as it gets a chance to feed on late larvae (AVEDEJEVA, 1979) or the larval food (DE JONG et al., 1982) of bees.

There are no such parallel studies on the oocyte structure of Varroa in the bee colonies in tropical climate. It could be expected that such restricted state of oocytes does not exist under such conditions, where, the bee brood is at the disposal of the mite for reproduction, throughout the year.

During the main phase of vitellogenesis, a peculiar type of yolk uptake mechanism seems to be characteristic of Varroa oocytes. The passage and deposition of material into membrane limited amorphous masses in the ooplasm appears to be somewhat similar to FAWCETT, 1965, type II and III (in combination) absorption by cells. These type involve elevation of thin undulating folds that impound sizable droplets of fluid and active change in surface configuration of the cell membrane, rather than a coalescing of vesicles derived from micropinocytotic pits or coveolae. The surface enfolded projections, were first

discovered by WARREN LEWIS in 1931 for cell cultures and latter discussed by FAWCETT, 1965, in intact endothelial cells of blood capillaries. It has been suggested that their function is to entrap the fluid by movements of the undulating membrane. It is conceivable, that it may become an important mechanism under more extreme conditions, requiring shifts of unusually large amounts of fluid across the cell membrane (FAWCETT, 1965). However, such bulk processes do not explain the selective uptake by specialized cells like vitellogenic oocytes. Nevertheless, the discovery of mucopolysaccharide coating over the free surfaces of nearly all such cells, suggested suitable properties for selective binding of particular class of ions or molecules to the cell surface, prior to the uptake into the cytoplasm (FAWCETT, 1965).

Such type of fold-like projections (microlamellae) of oolemma of Varroa oocytes is a specialized adaptation for rapid uptake of vitellogenins, enabling the mite to lay eggs quickly within the short time available in the capped brood cells. These microlamellae were more often found filled with electron dense material (when cut across) similar to the yolk sphere inclusions.

This specialization of oocyte surface appears to exist for a very short time, since it was found in an extremely low percentage of the mites used for this study. Perhaps this was the reason why WHITE, 1975, in Erythraeides and MOTHES and SEITZ, 1981, in Tetranychus urticae could not observe any micropinocytosis or other uptake mechanisms on the surface of the oocytes. Hence, they suggested a simple diffusion of yolk precursors (low molecular substances) directly either from hemolymph or midgut epithelium into the vitellogenic oocytes, as a simple explanation for such a complex process.

D. 3. Significance of nutrition in the reproduction of Varroa.

The role of nutrition in the reproduction of Varroa mite appears to be very significant. The high rate of reproduction and the intensity of spread and damages to the Apis mellifera colonies could be explained on this basis along with the other factors.

Reproductive success of a parasite is one of the most important determining factors for a successful continuation of population. Varroa

mite has recently achieved this aspect with success in Apis mellifera colonies. However, it has failed to achieve the host-parasite balance in this bee, in contrast to original host Apis cerana. The utilization of host proteins for the oogenesis of a parasite shows a high degree of adaptation. Perhaps the short period available for reproduction in the capped honey bee brood cells is an effective selection pressure which causes rapid development in all preadult stages, including oogenesis. The present functional study of nutritional and reproductive characteristics and adaptations of Varroa jacobsoni supports the above facts.

SUMMARY

The acarid, Varroa jacobsoni is an established ectoparasitic stenophagous mite of the eastern honey bee, Apis cerana. Recently, it has been transmitted to the western honey bee, Apis mellifera, where it is imparting enormous damage; so much so that, in cases of heavy infestations, whole bee colonies have been killed. The established host-parasite balance attained in A. cerana fails in the case of A. mellifera. The female mite and the nymphal stages feed on the hemolymph of late larval, pupal, and adult honey bees.

Up to now, neither attempts for a chemical solution nor for biological control have met with any success. The two main problems encountered in controlling the mite are: 1) that most of the acaricides leave residue either in the wax or in the honey and 2) that it is not possible to eliminate all the mites from a bee colony, because the female reproductive mite enters the honey bee brood cell just before the late larvae are sealed by worker bees for further development. The mite so enclosed in the cell, lays eggs and the nymphs start feeding on hemolymph from the corresponding stages of the bee. The chemotherapeutics are unable to reach them there.

There have been other systemic control methods in which the agents are fed to the bees and when the mite sucks the hemolymph it obtains enough substance to be fatal to it. However, such methods and other biological controls require precise information concerning the biology of the parasite. The failure to combat the Varroa problem is due to the fact that there are very few studies done with the intent of understanding the host-parasite relationship, the development of the mite and physiology of nutrition and reproduction. This study, therefore, was undertaken to collect data concerning questions on nutrition and reproduction in Varroa.

Honey bee hemolymph is the only food available to nymphal and adult mites during their reproductive cycle which is spent in the capped brood cells. This is the first time that the fate of honeybee (host) hemolymph proteins in Varroa (parasite) has been followed using various immunological, immunocytochemical, electrophoretical, and histological methods along with enzyme assays. The volume of meal consumed by

different age groups of mites and the total hemolymph volume of the host larvae (honey bee late larvae on which the reproductive female mite feeds before egg laying) was determined by isotope dilution method. These data are extremely valuable, especially for the application of systemic controls to the mite. From these studies it was concluded that the Varroa mite ingests the honey bee hemolymph proteins into its own hemolymph as whole macromolecular proteins, without any significant digestive degradation. A conclusion supported by the enzyme assays of Varroa proteolytic enzymes. Only exocarboxypeptidase-A was recognizable with the help of synthetic substrates. This enzyme equipment of the mite is not enough to cleave the long polypeptide chains of host blood. Not only host but experimentally offered vertebrate protein (BSA) was found immunologically intact in the mite's hemolymph. At the same time, immunocytochemical localization of these host proteins in the Varroa tissue makes it evident that this mite has reached a high degree of parasitic adaptation.

The role of these resorbed host proteins in the mite's hemolymph was followed further. The correlation between reproduction, especially oogenesis, and nutritional physiology was revealed when these foreign proteins were detected in the developing oocytes and in the freshly laid eggs using the above mentioned methods.

Also of great interest was the fact that the Varroa eggs contain hemolymph proteins which can only be taken during vitellogenesis. Using SDS-PAGE, 7 Apis hemolymph proteins and 1 of Varroa's own hemolymph proteins were identified as vitellogenins. The histological studies with the help of light and electron microscopy, showed several unique features of oogenesis in Varroa.

The utilization of foreign proteins (host) for Varroa's egg development indicates a high degree of parasitic adaptation. Perhaps the short period which is available for completing the whole life cycle in the capped bee brood is an effective selection pressure for rapid oogenesis in the mite.

The results presented here point in the direction of a high and very specialized parasite-host relationship in the physiology of nutrition, reproduction, and development in this mite. The degree of dependence from special host sources apparently is the result of a

long period of adaptation by which a high efficiency of reproduction in the mite was obtained by preferentially parasitizing drone bees and thereby avoiding hazard to the host, because a bee colony is mainly dependent on worker functions, but has a surplus of males. The harm caused in A. mellifera colonies by Varroa is probably due to the shift of the specific host selection behaviour of the reproductive female mite, which also accepts worker brood for reproduction. More studies are necessary to fully understand the underlying mechanism and consequences.

LITERATURE

Alberti, G.

Fortpflanzungsverhalten und Fortpflanzungsorgane der Schnabelmilben (Acarina: Bdellidae, Trombidiformis). Z.Morph.Tiere, 78, 111-157 (1974).

Atwal, A.S. and Dhaliwal, G.S.

Some behavioural characteristics of Apis indica F. and Apis mellifera L.
Indian Bee J. 31, 1-8 (1969).

Atwal, A.S. and Goyal, N.P.

Infestations of honey bee colonies with Tropilaelaps and its control.
J.Apic. Res., 10, 137-142 (1971)

Avedejeva, O.I.

Varroasis in bees. Cited without details by Grobov, O.F.
46-90 (1977)
From Varroasis-a honey bee disease., Bucharest,
Romania: Apimondia Publishing House.

Bailey, L.

Honey bee Pathology.
Academic Press Inc. (London) LTD. (1981)

Brem, S.

Laboruntersuchungen von Wittergemüll.
Diagnose und Therapie der Varroatose- Bucharest,
Romania: Apimondia Publishing House., 116-117 (1980).

Brinton, L.P. and Oliver, H.H.

Fine structure of oogonial and oocyte development in Dermacentor andersoni stiles (Acari_ Ixodidae).
J. Parasitol. 57, no. 4, 720-747 (1971).

Dahlman, B., Jany, K.-D. and Pfleiderer, G.

The midgut endopeptidases of honey bee (Apis mellifica):
Comparison of the enzyme in different ontogenic stages.
Insect Biochem., 8, 203-211 (1978)

Dalfinado, M.D. and Baker, E.W.

Tropilaelaps, a new genus of mites from the Phillipines
(laelapidae (S.Lat.): Acarina).
Fieldiana Zool., 44, 53-56 (1961).

Dalfinado, M.D.

Mites of the honey bees in Southeast Asia.
J. Apic. Res., 2, 113-114 (1963)

Dalfinado, M.D. and Baker, E.W.

Varroidae, a new family of mites of honey bees
(Mesostigmata: Acarina).
J. Wash. Acad. Sci., 64, 4-10 (1974).

De Jong, D. and Goncalves, L.S.

The Varroa problem in Brazil.
Amer. Bee J., 121, 186-189 (1981).

De Jong, D., De Jong, P.H. and Goncalves, L.S.

Weight loss and other damage to developing worker honey
bees from infestation with Varroa jacobsoni.
J. Apic. Res., 21, no. 3, 165-167 (1982).

De Jong D., Morse, R.A. and Eickwort, G.C.

Mite pests of Honey bees.
Ann. Rev. Entomol., 27, 229-252 (1982).

De Ruijter, A.

Geschlechtsdetermination bei Varroa Milben.
Arbeitsgemeinschaft der Institute für Bienenforschung
(15-17 March), Hohenheim, W.Germany. (1983).

Dumont, J.N. and Anderson, E.

Vitellogenesis in the horseshoe crab, Limulus polyphenus.

J. Microsc., 6, 791-806 (1967).

Engels, W.

Untersuchungen zur Dotter-protein-Haushalt der Honigbiene (Apis mellifica).

Wilh. Roux. Arch., 171, 55-86 (1972).

Erlandsen, S.L., Parson, J.A. and Rodning, C.B.

Technical Parameters of Immunostaining of Osmicated Tissue in Epoxy Sections.

J. Histochem. & Cytochem., 27, no. 9, 1286-1289 (1979).

Erlanger, B.F., Kokowsky, N. and Cohen, W.

The preparation and properties of two new chromogen substrates of trypsin.

Arch. Biochem. Biophys., 95, 271-278 (1961).

Erlanger, B.F., Edel, F. and Cooper, A.C.

The reaction of chymotrypsin on two new chromogenic substrates.

Arch. Biochem. Biophys., 115, 206-210 (1966).

Fawcett, D.W.

Surface specialization of absorbing cells.

J. Histochem. & Cytochem., 13, no.2, 75-91 (1965).

Grobov, O.F.

Varroasis in bees. 49-90 (1977).

From Varroasis- A honey bee disease., Bucharest, Romania: Apimondia Publishing House.

Hagedorn, H.H., Kunkel, J.G.

Vitellogenin and vitellin in insects.

Am. Rev. Entomol., 24, 475-505.

Haragsim, A.

Die Milbe Varroa jacobsoni Oudemans bedroht die Bienen-
zucht in Europa.

Imkerfreund, 28, 316-317 (1973).

Henking, H.

Beiträge zur Anatomie, Entwicklungsgeschichte und
Biologie von Trombidium fuliginosum Herm.

Z. Wiss. Zool, 37, 553-663 (1882).

Hirst, S.

On the mite Acaripis woodi, associated with the Isle
of White disease.

Ann. Mag. Nat. Hist., 7, 509-519 (1921).

Houk, E.J. and Hardy, J.L.

In-vivo negative staining of the midgut continuous
junction in the mosquito, Culex tarsalis (Diptera:
Culicidae).

Acta Tropica., 36, 267-275 (1979).

Ionescu-Varo, M. and Suciu, M.

Präliminarangaben über Anatomie and Histochemie der
Milbe Varroa jacobsoni Oudemans.

41-55 (1974)

From-Bekämpfung und Vorbeugung der Varroatose.,

Bucharest, Romania: Apimondia Publishing House.

Hughes, T.E.

Mites or the Acari.

Univer. of London., The Athlone Press, London.

Jany, K.-D.

Studies on the digestive enzymes of the stomachless
bonefish, Carassius auratus gibelio (Bloch): Endopeptidase.

Comp. Biochem. Physiol., 53B, 31-38 (1976).

Jenni, L.

Synthese und Aufnahme von Proteinen während der Vitellogenese in Oocyten von Ornithodorus maubata, Murry. Acta Tropica., 28, 105-163 (1971).

Klumpp, W.

Embryologie und Histologie der Bienenmilbe, Acaripis woodi, Rennie.

Z. für Parasitenkunde, 16, 407-442 (1954).

Koeniger, N., Koeniger, G. and Wijaygunasekara, N.H.P.

Beobachtungen über die anpassung von Varroa jacobsoni an Ihren natürlichen wirt Apis cerana in Srilanka.

Apidologie, 12, 37-40 (1981).

Krøll, J.

Tandem-crossed Immunelectrophoresis.

From- A manual of Quantitative Immunelectrophoresis.

Scand. J. Immunol., 2, Suppl. no. 1, 57-59 (1973).

Laemmli, U.K.

Cleavage of structural protein during the assembly of the Head of Bacteriophage-T₄.

Nature, 227, 680-685 (1970).

Langhe, A.B. and Natskii, K.V.

The mite Varroa and the methods of controlling it. 40-46 (1977).

From- Varroasis-A honey bee disease., Bucharest, Romania: Apimondia Publishing House.

Lange, A.B., Talzij, W.M. and Natzkij, K.W.

Opredele nije stepeni poraschenija ptsche Varroatosom.

Plschelowodstwo, 3, 25 (1978).

Lewis, W.H.

Pinocytosis

Bull. Johns Hopkins Hosp., 49, 17-27 (1931).

Mancini, G., Carbonara, A.O. and Havemans, F.J.

Immunochemical quantitation of antigens by single radial diffusion.

Immunocytochemistry, 2, 235 (1965).

Moritz, R.F.A.

Altersabhängige Empfindlichkeit von Varroa jacobsoni Oudemans gegen K-79 (Chlorodimeformhydrochloride). 62-68 (1981).

From-Diagnose und Therapie der Varroatose., Bucharest, Romania: Apimondia Publishing House.

Morse, R.A.

Asian bee mites.

Glean. Bee Cult., 97, 287-315 (1969).

Mothes, U. and Seitz, K.-A.

Feinstrukturelle und Autoradiographische Untersuchungen zur Oogenese von Cupiennius salei Keys. (Araneae, Ctenidae) und Tegenaria derhami (Araneae Agelenidae). Zool. Jb. Anat., 103, 133-152 (1980).

Mothes, U. and Seitz, K.-A.

Licht und Electronmicroscopische Untersuchungen zur Funktionsmorphologie von Tetranychus urticae (Acari, Tetranychidae). II. Weiblichen Geschlechtssystem und Oogenese.

Zool. Jb. Anat., 105, 106-134 (1981).

Michael, A.D.

On the variations in the internal Anatomy of the Gamasinae, especially in that of the Genital organs, and on their mode of Coition.

Tr. Linn. Soc. Lond. (Zool.) Ser. 2, 5, 281-317 (1892).

Nath, V., Gupta, B.L. and Rani, V.

Histochemical and morphological studies of the lipids in oogenesis. VII. The spider Crozzopriza lyoni (Black-wall).

Res. Punjab Univ., 154, 235 (1958).

Nedealkov, S.

Varroasis in Bulgaria, experience gained from controlling this bee parasitosis., 6-11 (1977).

From-Varroasis- A honey bee disease., Bucharest, Romania: Apimondia Publishing House.

Nogge, G.

On the absorption of undigested albumin by the tsetse fly, Glossina m. morsitans Westwood.

Proc. German Zool. Soc., 73rd Meeting, Berlin, 310 (1980).

Örösi-Pál

Experiment on the feeding habits of Acarapis mites.

Bee World, 15, 93-94 (1934).

Osaki, H.

Electron microscopic studies on the oocyte differentiation and vitellogenesis in the liphistiid spider, Heptathela kimurai.

Ann. Zool. Jap., 44, 185 (1971).

Ouchterlony, Ö.

Antigen-antibody reactions in gel.

Archiv Kemi. Mineral. Geol., 26B, no. 14, 1-9 (1948).

Pahnke, J.

Anatomisch-Biologische Untersuchungen an Limnochares aquatica.

L. Diss. Kiel, (1974).

- Pan. M.L., Bell, W.J. and Telfer, W.H.
 Vitellogenic blood protein synthesis by insect fat
 body.
 Science, 165, 393-394 (1969).
- Pojakow, A.A., Smirnow, A.M., Kulikowski, A.W. and Smirnowa, O.I.
 Electronmikroskopische Untersuchung des Bienen-
 schädlings Varroa jacobsoni (Oudemans, 1904).
 410-414 (1975).
 Internationaler Bienenzüchterkongress, Grenoble, Frank-
 reich.
- Rademacher, E.
 Laborversuche mit K-79 im Einsatz gegen Varroa jacob-
 soni.
 155-167 (1980).
 From-Diagnose und Therapie der Varroatose, Bucharest,
 Romania: Apimondia Publishing House.
- Reuter, E.
 Zur Morphologie und Ontogenie der Acaridae mit beson-
 derer Berücksichtigung von Pedicuopsis graminum (E.
 Reuter).
 Acta Soc. Sc. Fenn. Helsinki, 36, 1-288 (1909).
- Ritter, W.
 Zur Methodik der Prüfung von Chemotherapeutika zur
 Bekämpfung der Varroatose der Honigbienen.
 Apidologie, 11, (2), 131-141 (1980,b).
- Ritter, W.
Varroa disease of the honeybees, Apis mellifera.
 Bee World, 62, (4), 141-153 (1981).

Roth, T.F. and Porter, K.R.

Yolk protein uptake in the oocyte of Mosquito, Aedes aegypti.

L.J. Cell Biol., 20, 313-332 (1964).

Ruttner, F., Koeniger, N. and Ritter, W.

Brutstop und Brutentnahme.

Allg. dt. Imkerztg., 14, (5) 159-160 (1980).

Ruttner, F. and Ritter, W.

Das Eindringen von Varroa jacobsoni nach Europa im Rückblick.

ADIZ., 5, 130-133 (1980).

Ruttner, F., Ritter, W. and Götz, W.

Chemotherapie der Varroatose über die Hämolymphe der Biene.

ADIZ., 14, 160-166 (1980,b).

Schabanov, M., Neealkov, S. and Toshkov, A.

Varroatosis- a dangerous parasitic disease of bees.

Am. Bee J., 118, 402-403, 407 (1978).

Schulz, A.

Fortpflanzungsdynamik von Varroa jacobsoni.

Arbeitsgemeinschaft der Institut für Bienenforschung
(15-17 March), Hohenheim, W. Germany. (1983).

Schulz, F. and Koeniger, N.

Versuche zur biologischen Therapie der Varroatose durch
eine Kontrolle der frisch geschlüpften Bienen.

Apidologie, 11, (2), 105-112 (1980).

Smirnov, A.M. and Chernov, K.S.

Beekeeping in Japan.

Pchelovodstvo (2) 27-28; (3) 36-38; (4) 29-30; (5) 45-47 (in Russian) (1976).

Smirnov, A.M.

Aktuelle Ergebnisse der Sowjetischen Wissenschaft über Ätiologie, Pathologie, Epizootologie, Diagnose und Bekämpfung der Varroatose-, 60-74 (1970).

From-Bekämpfung und Verbeugung der Varroatose., Bucharest, Romania: Apimondia Publishing House.

Smirnowa, O.I., Machno, P.M., Simetzkij, A.M., Smirnow, A.M. and Kudrjawzew, E.P.

Mechanism dejstwija Varroatina na Kleschtscha Varroa. Ptschelowodstwo, 2, 8-11 (1978).

Steiner, J., Pompolo, S. das G., Takahashi, C.S. and Goncalves, L.S.

Cytogenetics of the Acarid Varroa jacobsoni.

Rev. Brasil Gent, 4, 841-844 (1982).

Sternberger, L.A., Hardy, P.H., Jr., Cuculis, J.J. and Meyer, H.G.

The unlabeled antibody-enzyme method of Immunocytochemistry. Preparation and properties of soluble antigen-antibody complex (Horseradish peroxidase-anti-horseradish peroxidase) and its use in identification of spirochetes.

J. Histochem. & Cytochem., 18, 315-333 (1970).

Sternberger, L.A.

Immunocytochemistry.

Engelwood Cliffs, N.J. Prentice-Hall, (1974).

Stort, A.C., Goncalves, L.S., Malaspina, O. and Moura Duarte, F.A.
Study on Syneacar effectiveness in controlling Varroa jacobsoni.

Apidologie, 12, (3) in Press. (1981).

Thomae, H.

Beiträge zur Anatomie der Halacariden.

Zool. Jb. Anat., 47, 155-190 (1926).

Thomson, E., Hansen, B.L., Hansen, G.N. and Jensen, P.V.

Ultrasturctural Immunocytochemical Localization of
Vitellogenin in the Fat Body of the Blowfly,
(Callifora vicina) Rob. Desv. (erythrocephala Meig.)
by use of the unlabeled Antibody-Enzyme Method.

Cell and Tiss. Res., 208, 445-455 (1980).

Von Frisch, K.

Aus dem Leben der Bienen.

Verständliche Wissenschaft, Springer-Verlag, (1977).

Wigglesworth, V.B.

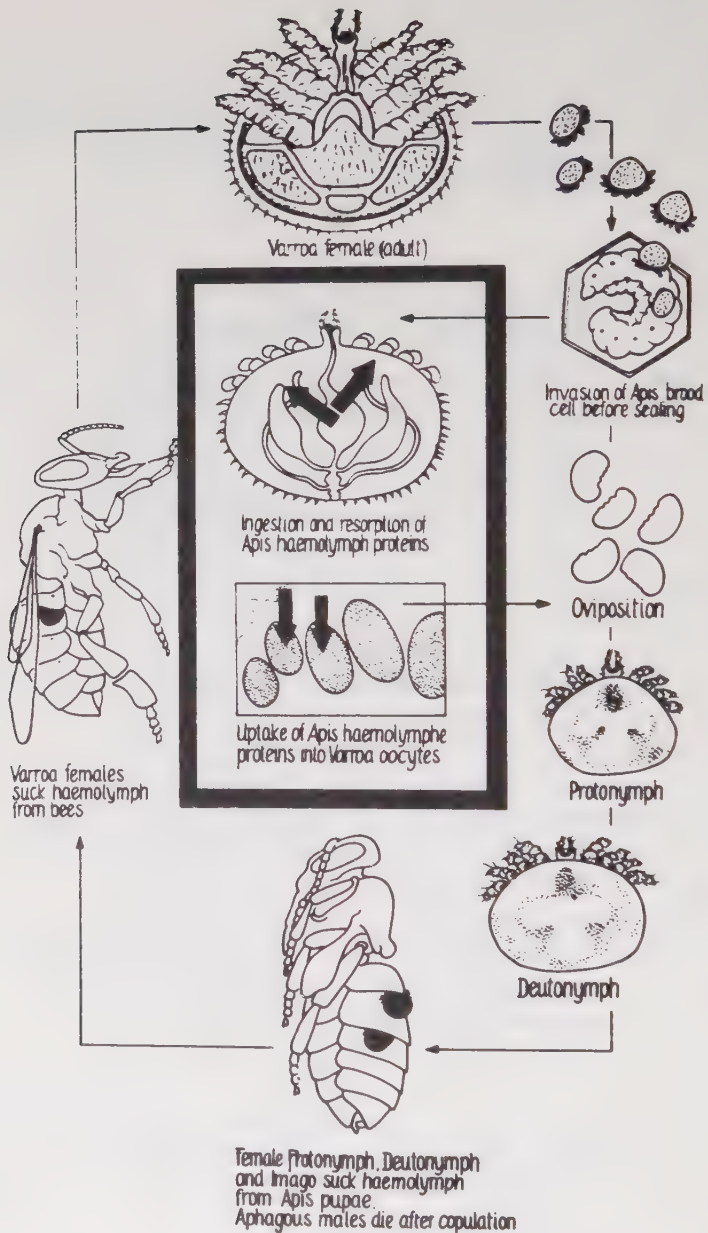
The fate of haemoglobin in Rhodnius prolixus
(Hemiptera) and other blood sucking Arthropodes.

Proc. R. Soc. Lond., Ser. B, 131, 313-339 (1943).

Witte, H.

Functionsanatomie des weiblichen Genitaltraktes und
Oogenese bei Erythraeiden (Acari, Trombidiformes).

Zool. Beiträge., 21, 247-277 (1974).



Life cycle of *Varroa jacobsoni* : The role of nutrition (*Apis* hemolymph proteins) in the development of the eggs.

			varroa female			short time			long time					
			0 h	6 h	24 h	3/4 h	1.5 h	3 h	6 h	12 h	24 h	36 h	48 h	
Young <u>Varroa</u> females	hatching workers	worker larvae	X	X	-	0.86 +0.03	-	0.90\$ +0.06	0.85 +0.02	-	-	-	0.80 +0.05	
	hatching drones	worker larvae	X	-	-	-	0.83	-	-	-	-	-	-	
	hatching drones	drone larvae	-	X	X	-	-	0.81\$ +0.02	-	-	-	-	-	
Old <u>Varroa</u> females	drone late larvae	drone larvae	X	-	-	0.83 +0.06	0.83 +0.03	0.85\$ +0.01	0.81\$ +0.03	0.87\$ +0.02	0.85\$ +0.01	0.83\$ +0.02	0.85\$ +0.01	
	drone prepupae	drone larvae	-	-	X	-	0.88 +0.04	0.86\$ +0.02	0.85\$ +0.03	0.87\$ +0.03	0.83\$ +0.02	-	-	
Gravid <u>Varroa</u> females	drone prepupae and pupae	drone larvae	X	-	X	0.83	0.84	0.87\$	-	0.82\$	-	-	-	
Old <u>Varroa</u> females	worker larvae and prepupae	nursing bees, 8 days old	-	X	-	-	-	-	-	0.85\$	-	-	-	
	EXCREMENTS			-			-	-	0.40	0.50	0.60	0.70	0.70	
No. of mites used for the experiment (Total 89)			21			15	26	2	17	3	2	3		

§ - (Excrements are not included)

TABLE 1.

Enzyme	Substrate (50 μ l)	Test-Enzyme (20 μ l)	<u>Varroa</u> Homogenate (20 μ l)	Activity % of Test-Enzyme
Overall Proteolytic Activity	Azocasein	+++ (§)	++	39.8
Overall Proteolytic Activity	Hide-Powder-Azure	+++	++	52.5

(§)

++ Average activity (30 - 80 %)

+++ Very high activity (30 - 100 %)

Mixture was prepared in Tris-HCl Buffer pH 8.2 and incubated for 1 - 3 h at 37°C.

TABLE 2. - Overall proteolytic activity in Varroa jacobsoni

Enzyme	Substrate (50 μ l)	Test-enzyme (20 μ l)	<u>Varroa-</u> Homogenate (20 μ l)	Activity % of Test-enzyme
Endopeptidases	N α Glutaryl-L-phenyl-alanin-4 (GPNA)	+++ (§)	+	2
Endopeptidases	N α Benzoyl-L-arginin-4Nitroanilide (BAPA)	+++	+	5

(§)

+ Very low activity (1 - 10 %)

+++ Very high activity (80 - 100 %)

Mixture was prepared in Tris-HCl buffer pH 8.2 and incubated for 1 - 3 h at 37°C.

TABLE 3. - Enzyme activity of endopeptidases present in Varroa jacobsoni against different artificial substrates

Enzyme	Substrate (50 μ l)	Test-enzyme (20 μ l)	<u>Varroa-</u> Homogenate (20 μ l)	Activity % of Test-enzyme
Carboxy - peptidase-B	Hippuryl-lysine	++ (§)	—	0
Carboxy- peptidase-A	Hippuryl-Phenyl- alanin	+++	+++	98.0
Aminopeptidase	L-alanin-4-nitro- anilide	+++	+	25.7

(§)

— No activity

+

++ Average activity (30 - 80 %)

+++ Very high activity (80 - 100 %)

Mixture was prepared in Tris-HCl buffer pH 8.2 and incubated for 1 - 3 h at 37°C.

TABLE 4. - Identification of exopeptidases in Varroa jacobsoni

Test No.	pitted trays	Varroa collected from	host locating time	total No. of tested mites	No. of mites dead or elsewhere	No. of mites on worker larvae (late L5)	No. of mites on drone larvae (late L5)	% preference for male larvae
1.	without wax	nursing bees	5 min.	7	-	2	5	71.4
2.	without wax	late larvae	10 min.	9	1	1	7	87.5
3.	without wax	nursing bees	10 min.	24	2	8	14	63.6
4.	wax coated	nursing bees	15 min.	9	2	2	5	71.4
5.	without wax	nursing bees	30 min.	13	1	4	8	66.6
6.	without wax	nursing bees	3 hrs.	10	2	2	6	75.0
7.	without wax	prepupae	3 hrs.	10	-	0	10	100
8.	without wax	pupae	6 hrs.	25	2	6	17	73.9
9.	without wax	nursing bees	12 hrs.	17	-	5	11	64.7
10.	wax coated	nursing bees	24 hrs.	8	1	3	4	57.1
11.	without wax	prepupae	24 hrs.	16	2	6	8	57.1
12.	without wax	nursing bees	24 hrs.	20	1	6	13	68.4
13.	without wax	nursing bees	26 hrs.	28	6	6	16	72.7
14.	wax coated	late larvae	36 hrs.	12	-	2	10	83.3

Total 208

$\bar{X}$ - 72.34
SD $\pm$ 11.68

range: 57.1 - 100.0



Fig.I. Adult male Varroa j. mite, ventral side, Rasterelectronmicrograph. Magnification: see bar in the Fig.

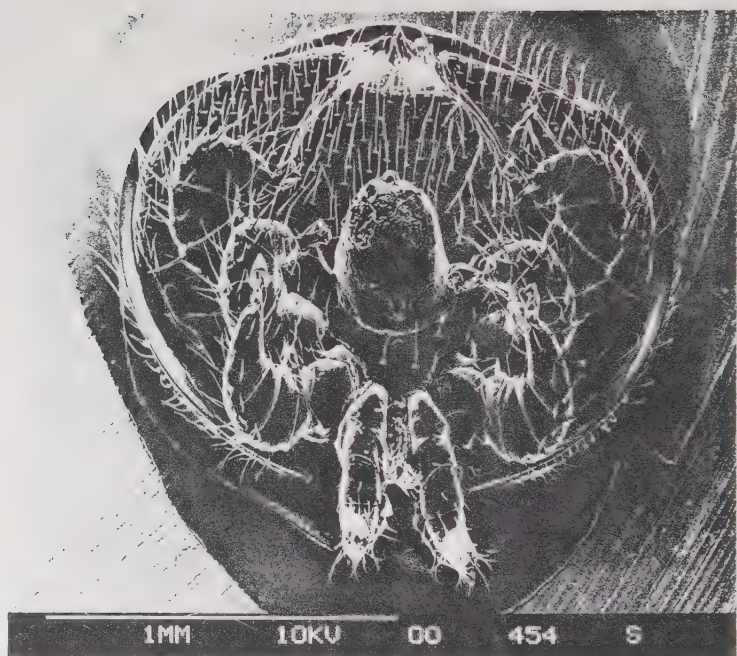


Fig.II. Adult female Varroa j.mite, ventral side. An egg is seen in the oviposition still attached near the genitoventral plate. Note the glue (darkish), a product of the accessory glands, with the help of which it is attached to the honey bee wax cell wall. Rasterelectronmicrograph. Magnification: see bar in the Fig.

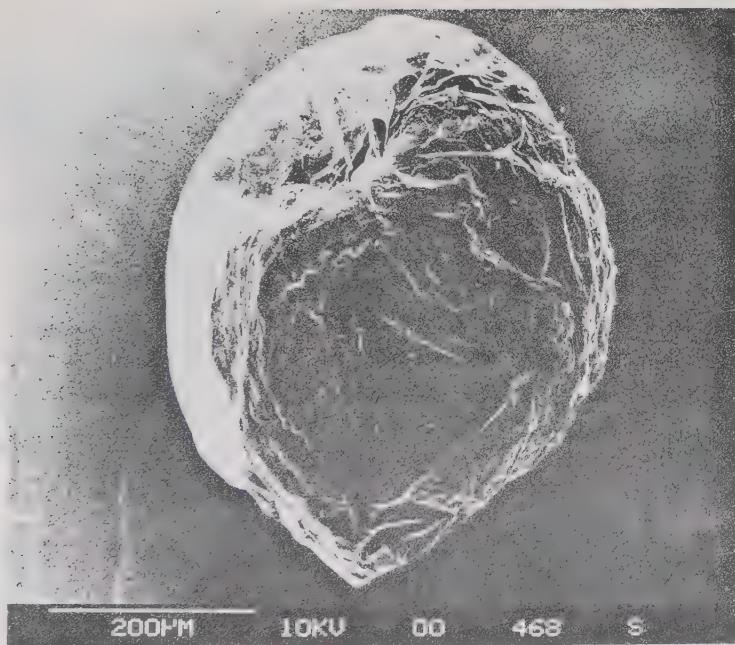


Fig.III. An egg of Varroa j.mite. The little shrinkage of the egg shell is caused during fixation. Rasterelectronmicrograph. Magnification: see bar in the Fig.

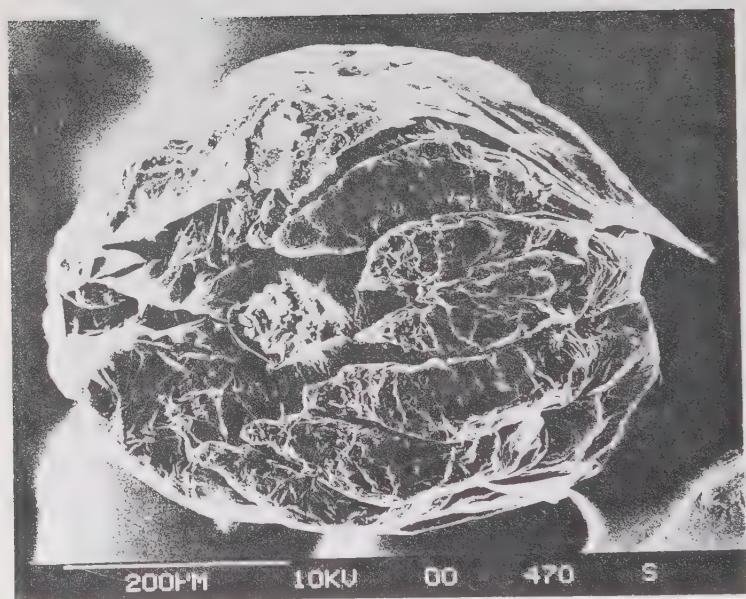


Fig.IV. The nymph of Varroa j. mite, just hatching out of the egg shell. The mouth parts and appendages are already developed. Raster-electronmicroscope. Magnification: see bar in the Fig.

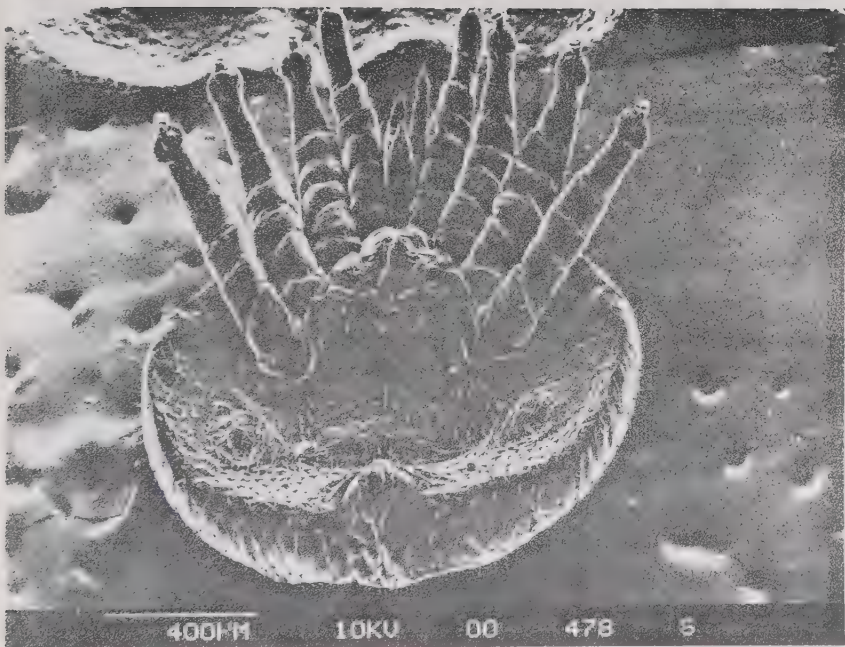


Fig.V. Protonymph of Varroa j. mite, Ventral view. Rasterelectron-
micrograph. Magnification: see bar in the Fig.

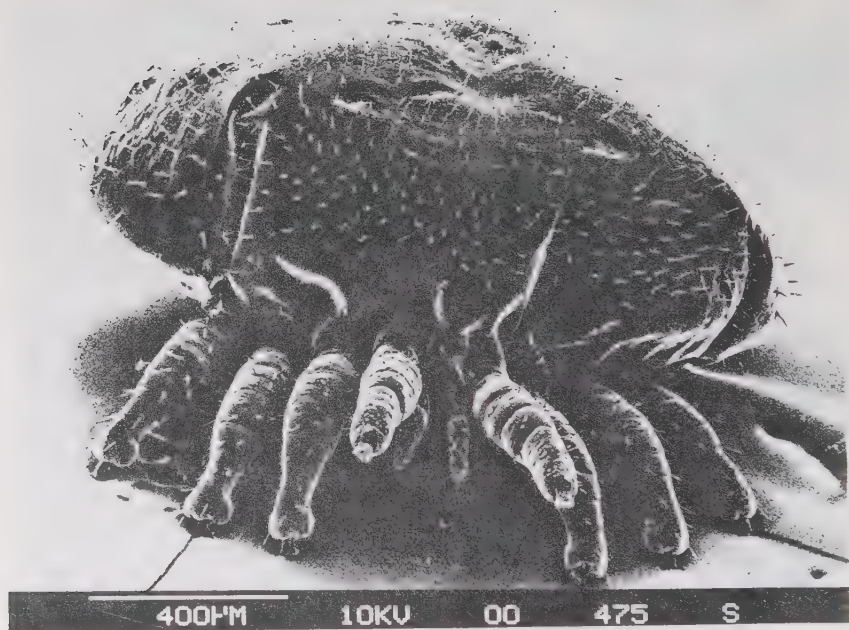


Fig.VI. Deutonymph of Varroa j.mite, frontal view. Shrinkage in exuvia is caused during the fixation. Magnification: see bar in the Fig.

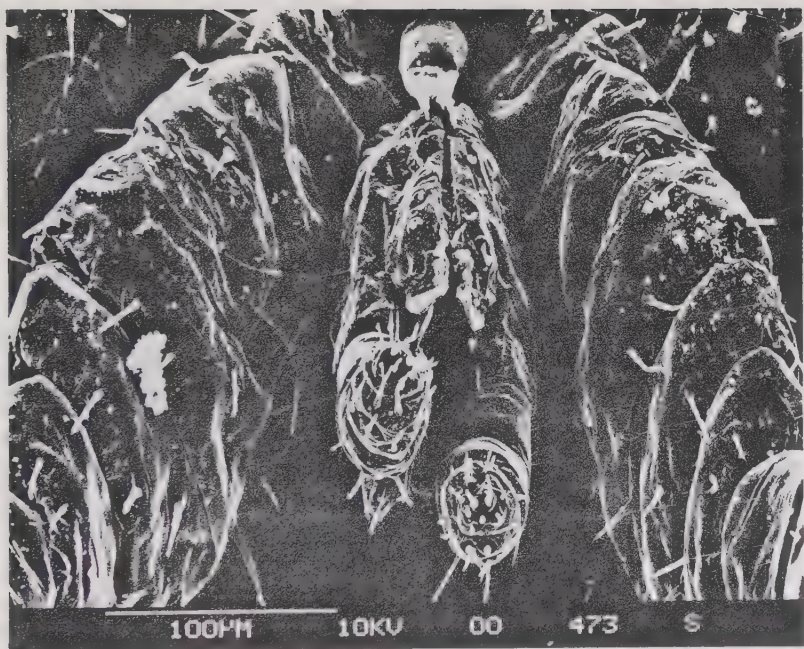


Fig.VII. The ventrofrontal portion of the Gnathosoma of Varroa j. female mite. It shows the first pair of legs, pedipalpi and the chelicerae modified for drilling and sucking type.

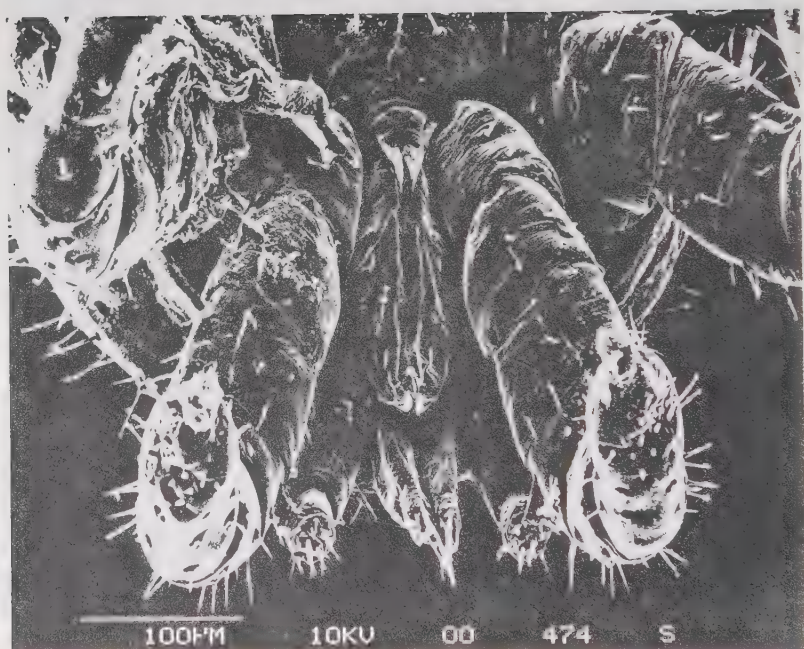
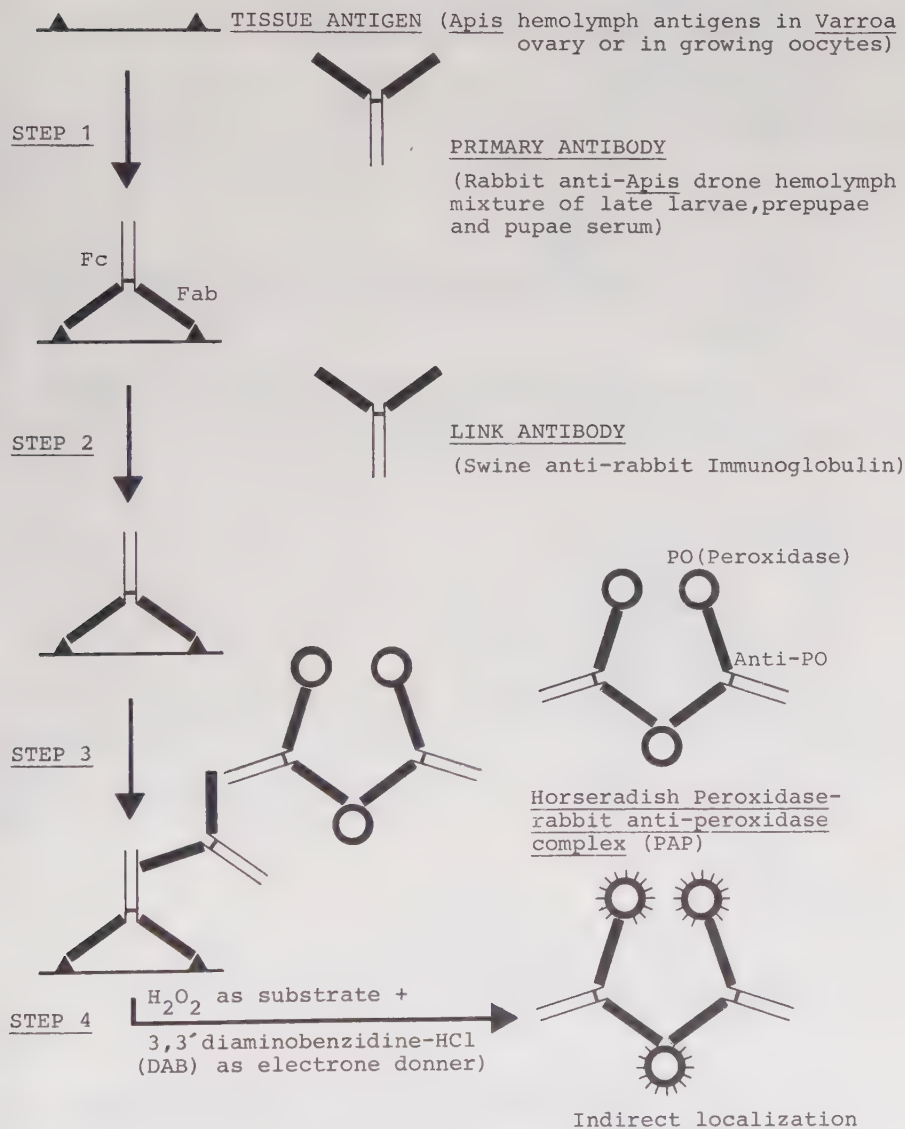


Fig.VIII The ventro-frontal portion of the gnathosoma of Varroa j. male mite. The rasterelectronmicrograph shows two pairs of legs, pedipalpi similar to female mite, however, the chelecrae in the male mite are modified into sperm transforming organs. Magnification: see bar in the Fig.

Fig.1

IMMUNOCYTOCHEMICAL LOCALIZATION OF APIS HEMOLYMPH PROTEINS
IN THE OOCYTES OF VARROA JACOBSONI

(Unlabeled antibody-enzyme method,PAP):STERNBERGER et al.,1970



CHANNELGROUP -1

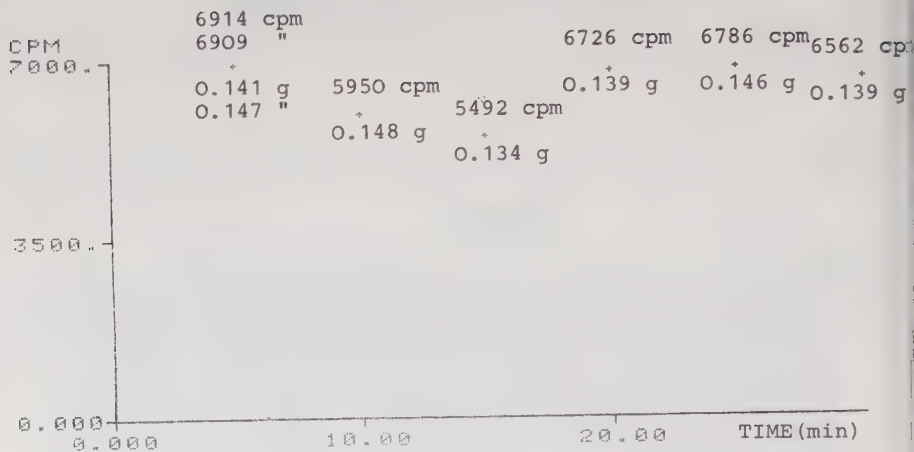


Fig. 2 a: Scatter diagram plotted against the time in min. for calculating the hemolymph volume of the Apis worker larvae.

CHANNELGROUP -1

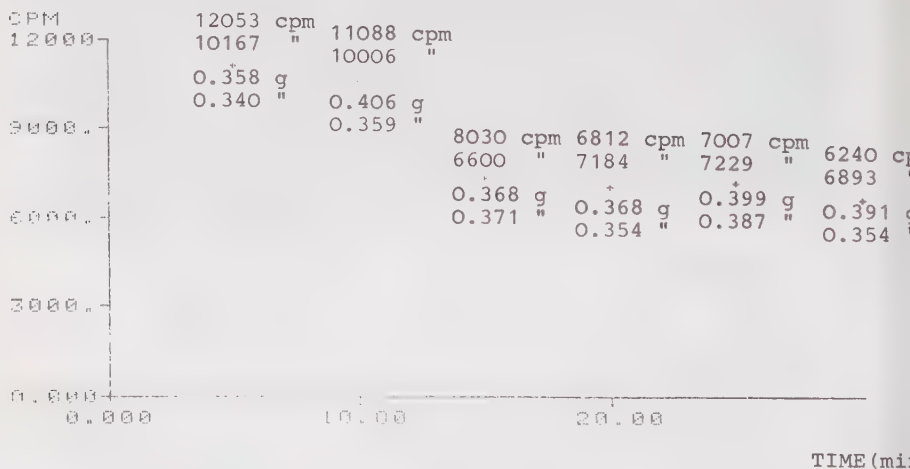


Fig. 2 b: Scatter diagram plotted against the time in min. for calculating the hemolymph volume of the Apis drone larvae.

CHANNELGROUP -1

ABS. FREQUENCY

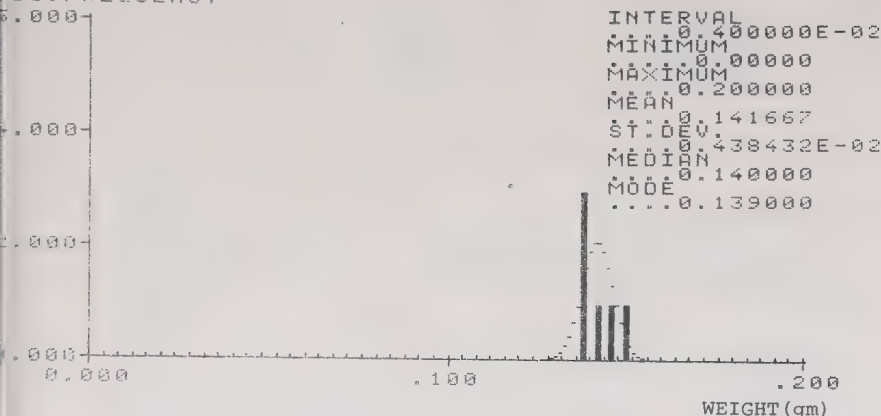


Fig. 2 c: Gaussian's distribution of the weight of the Apis worker larvae to correlate with hemolymph volume determination.

CHANNELGROUP -1

ABS. FREQUENCY

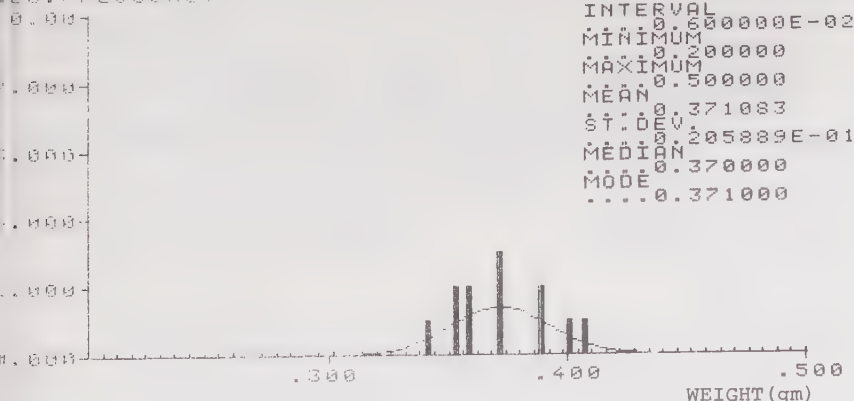


Fig. 2 d: Gaussian's distribution of the weight of the Apis drone larvae to correlate with hemolymph volume determination.

CHANNEL GROUP 1

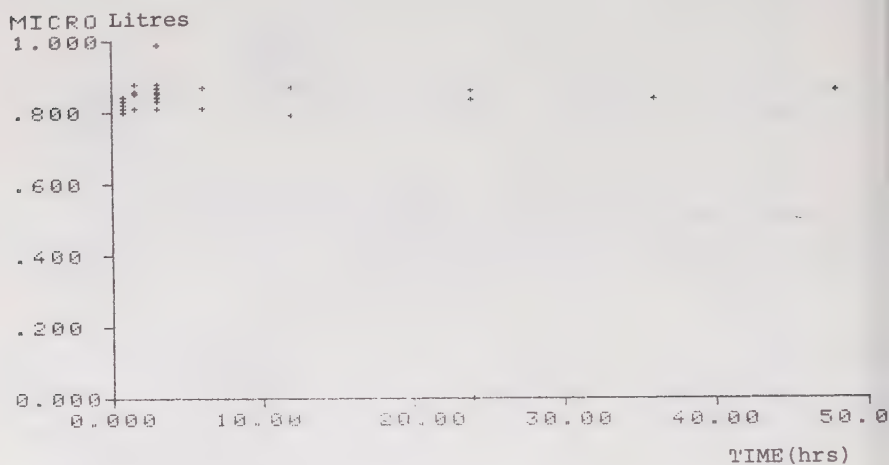


Fig. 3: Scatter diagram plotted for the Apis (host) hemolymph volume consumed by Varroa female (parasite) in μ ls. verses the time in hrs. of feeding (without considering the excrements, deposited during these intervals).



Fig.4. Detection of Apis hemolymph proteins in the hemolymph of Varroa nymphs and females.

Radial immunodiffusion in antibody impregnated agarose gel. AD- antiserum of rabbit against Apis drone hemolymph mixture of late larvae, prepupae and pupae; D-hemolymph of Apis drone pupae; v- hemolymph of female Varroa; n- hemolymph of Varroa nymphs.

The size of the precipitin rings are directly proportional to the concentration of the antigen used. In all the immuno-tests capital letters are used for the Apis and small for Varroa samples.

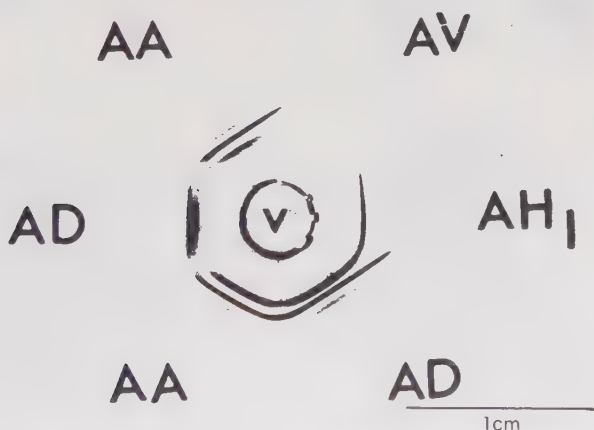


Fig.5. Cross reaction of Varroa hemolymph proteins with different antibodies directed against the Apis hemolymph proteins.

Double-immunodiffusion in agarose gel. v- hemolymph of Varroa females; AA- antibody against Apis worker prepupae hemolymph; AD- antiserum against drone hemolymph mixture of late larvae, prepupae and pupae; AV- antiserum against Apis separated vitellogenin; AH₁- antiserum against separated Apis hemolymph proteins fraction H₁.

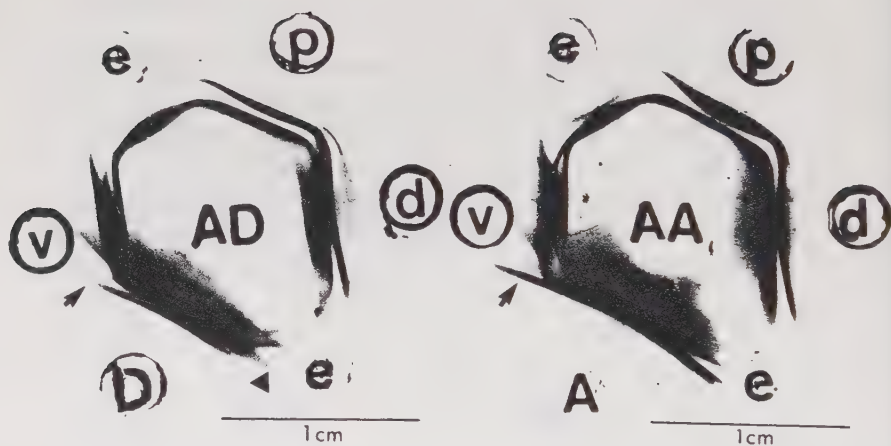


Fig.6 a&b. Detection of Apis hemolymph proteins in the hemolymph of Varroa female, protonymph, deutonymph and egg homogenate. Double-immunodiffusion in agarose gel. AD- and AA- compare Fig.5; v- hemolymph of Varroa females; e- Varroa egg homogenate; p- hemolymph of mites protonymphs; d- hemolymph of deutonymphs; D- hemolymph of Apis drone pupae; A- hemolymph of worker. Spur formation ($\rightarrow$, $\blacktriangleright$) showing a little change in antigenic determinants during the passage through the Varroa midgut.

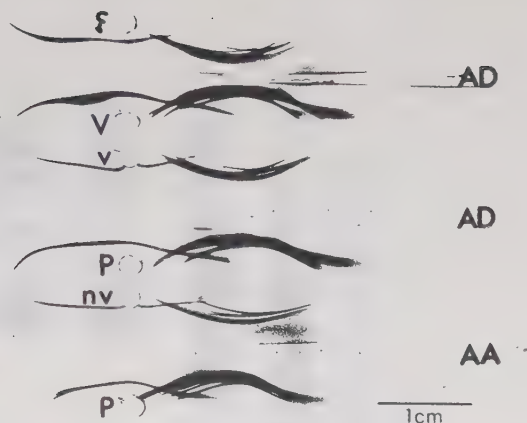


Fig. 7: Detection of host (Apis) hemolymph proteins in the hemolymph of Varroa female and nymphs.

Immunoelectrophoresis in agarose gel. S-hemolymph of Apis late larvae; V-hemolymph of Apis prepupae; P-hemolymph of pupae; v-hemolymph of Varroa females; nv-hemolymph of Varroa nymphal stages; AD-and AA- compare Fig. 5

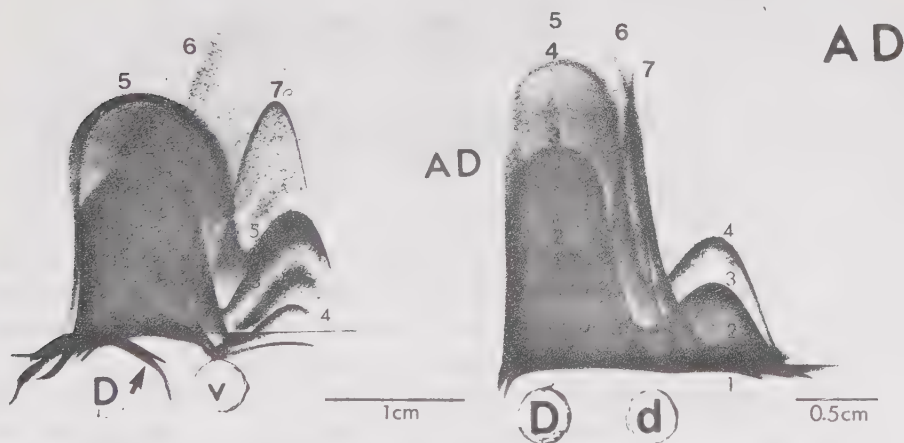


Fig. 8 a & b: Detection of Apis hemolymph proteins in the hemolymph of female Varroa and deutonymph.

Two dimensional Tandem-cross-immunoelectrophoresis in antiserum impregnated agarose gel. D-hemolymph of Apis drone pupae; v-hemolymph of Varroa females; d-hemolymph from deutonymph; AD-antiserum against Apis drone hemolymph mixture of larvae, prepupae and pupae. (big numbers are used for Apis and small numbers for Varroa samples) (→) shows some subsidiary fractions in drone hemolymph sample in Fig. 8a.

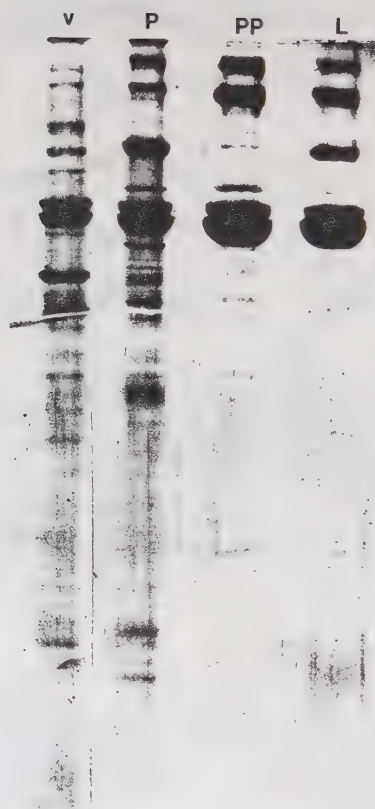


Fig. 9: Electrophoretic detection of Apis hemolymph proteins in the hemolymph of female Varroa in SDS-polyacrylamide slab gel electrophoresis in 15 % resolving gel. v-hemolymph of Varroa females; PP-hemolymph of prepupae; L-hemolymph of late larvae.

The separation patterns of mite and bee stages hemolymph clearly shows the full identity of almost all the proteins, confirming the earlier immunological findings that the host blood proteins are resorbed by the mite and its nymph in their own hemolymph without significant digestive degradation.

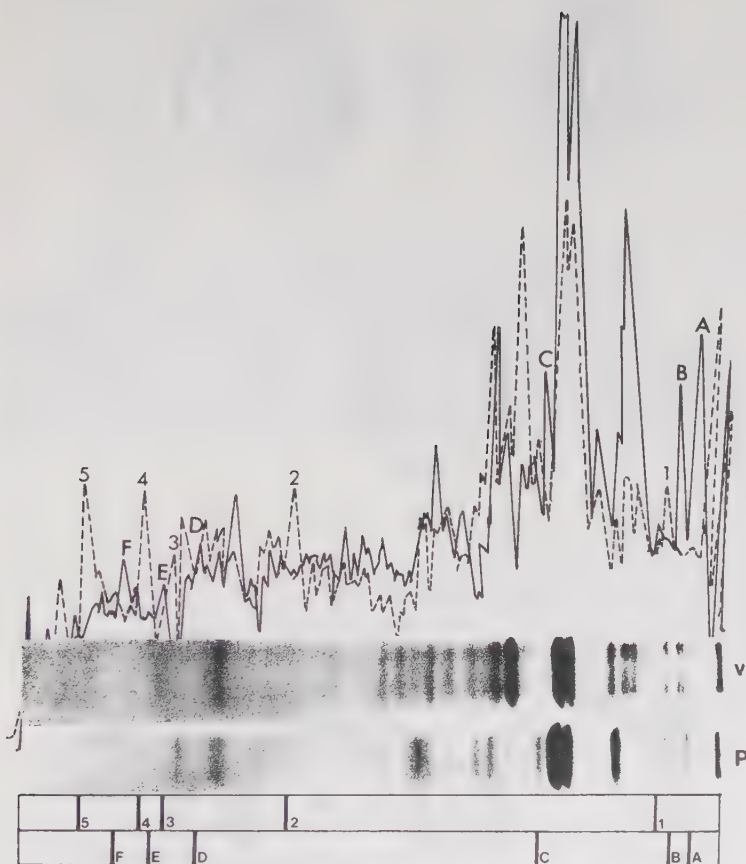


Fig. 10: SDS-PAGE scanning patterns: The figure shows the gel scanning patterns of the same gel shown in Fig. 9, however, only Apis pupal hemolymph (——) and Varroa females (----) hemolymph samples are compared. 1 - 5 Varroa own hemolymph proteins are distinguishable and A - F Apis hemolymph proteins are absent in the hemolymph of Varroa, indicating their proteolytic degradation during resorption.



Fig. 11: Detection of Apis larvae food proteins in Varroa mite.

Radial immunodiffusion in antiserum impregnated agarose gel. D-larval food of Apis drone larvae; R-royal jelly; v-Varroa females homogenate; AR-antiserum against honey bee royal jelly proteins. The precipitin disc size is dependent on the antigen concentrations of the samples.

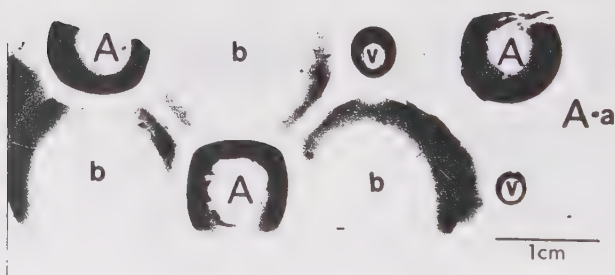


Fig. 12: Detection of undigested albumin (BSA) in the hemolymph of Varroa.

Radial immunodiffusion in the 10 % antiserum impregnated 1 % agarose gel. A-hemolymph of BSA injected nurse stage bees; b-BSA solution; v-pooled hemolymph of Varroa females, which fed on the injected bees.

The disc sizes are directly proportional to the protein concentration of the samples.

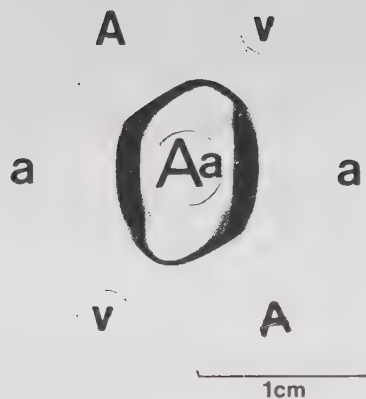


Fig. 13: Detection of undigested albumin (BSA) in the hemolymph of Varroa.

Ouchterlony double-immunodiffusion test for albumin contents of Varroa and Apis blood against the anti-albumin serum in 1 % agarose gel. Aa-anti-bovine albumin serum; A-Apis worker hemolymph from the BSA injected nurse stage bees; v-hemolymph of Varroa females which had fed on the BSA injected bees; a-BSA solution. The precipitating ring without any spur formation points out the presence of the BSA in all the samples.

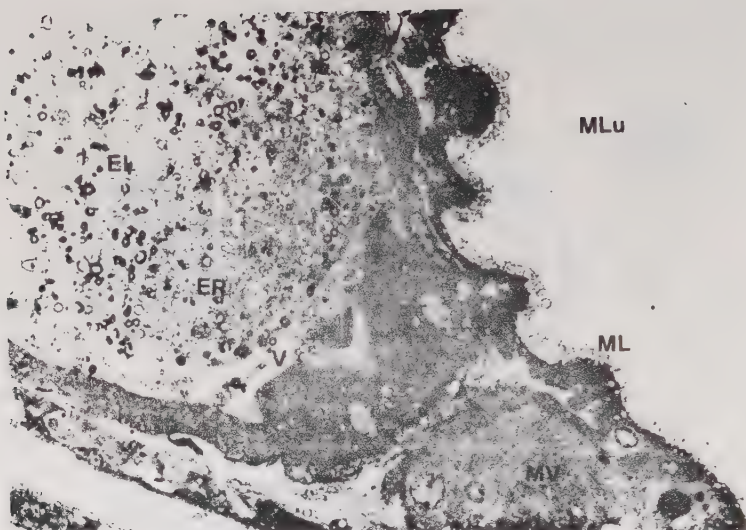


Fig. 14: Semithin Epon cross section of Varroa female, showing the midgut or ventricular coecum and excretory tube.
 MV-midgut coecum; ML-microlamellae; V-microvilli; EL-lumen of excretory tube; MLu-midgut lumen; ER-excretory products. Section is Toludin blue stained. Magnification: 1000 x.

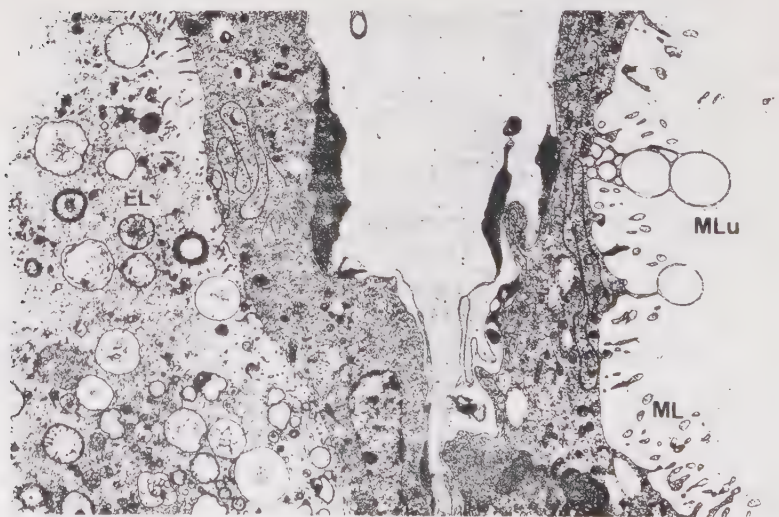


Fig. 15 a: Electronmicrograph (3000 x) of the same section as in Fig. 14, showing a part of midgut coecum and excretory tube.

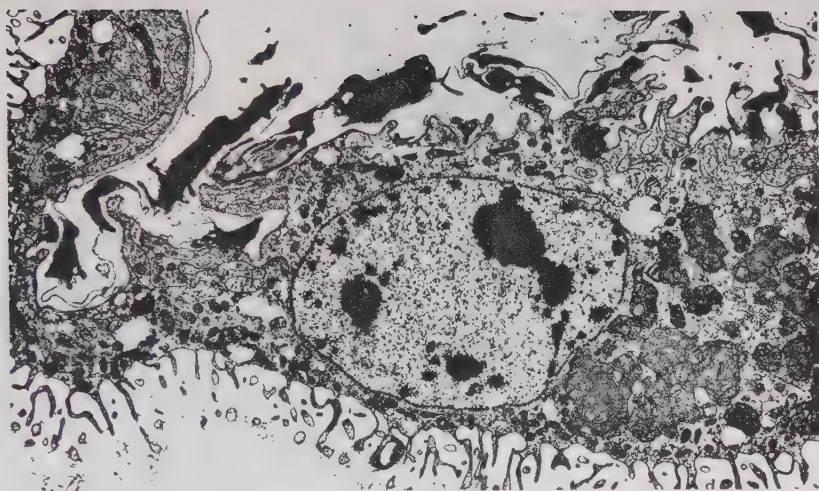


Fig. 15 b: Electronmicrograph (3000 x) of a cross section of a female Varroa, showing excretory tube.

The microvilli, continuous junction, mitochondria, nuclei of the epithelial cells, basement membrane and in the lumen excretory products are visible.

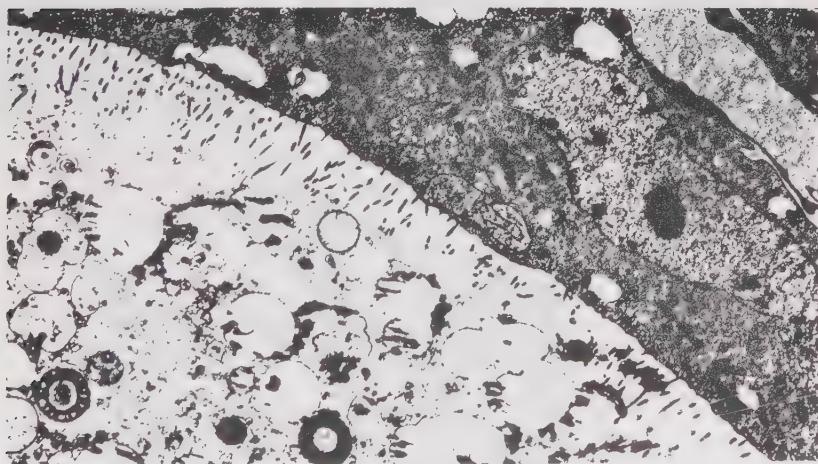


Fig. 15 c: Same section as above, but showing the midgut coecum.

The microlamellae , endocytotic pits, vesicles, mitochondria, nucleus with chromatin material, plasma membrane compartmentalization towards the hemocoel, basement membrane are seen along with endocytotic pits. The characteristic feature of the midgut coecum is the presence of microlamellae.

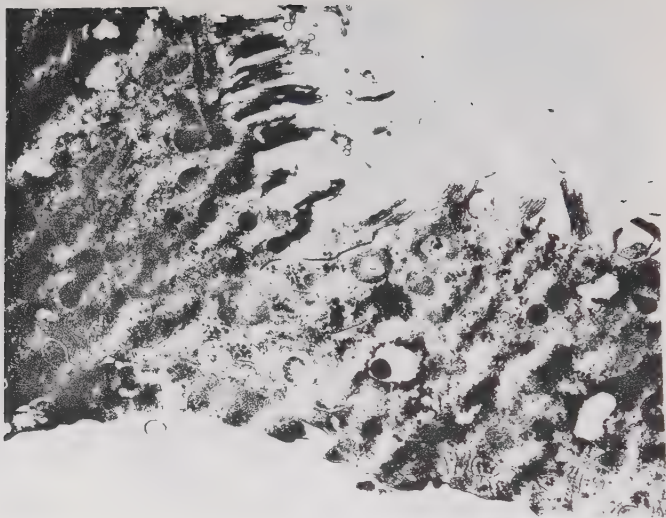


Fig. 15 d: Electronmicrograph (4700 x) of the female Varroa mite, showing the part of the midgut coecum. From exterior to interior basement membrane which also includes exocytotic pits, empty and electron dense material filled vesicles, mitochondria, continuous junction, endocytotic pits. The section shows the unusual brush border of midgut epithelium, having plate like foldings which are termed as microlamellae.

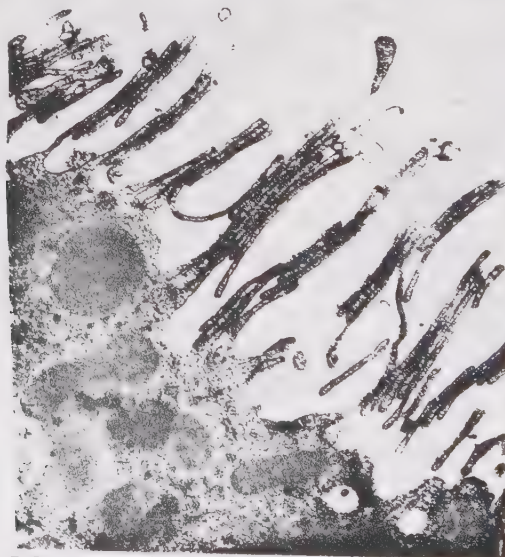


Fig. 15 e: Enlargement (9500 x) of the same above section showing the contour of the microlamellae.

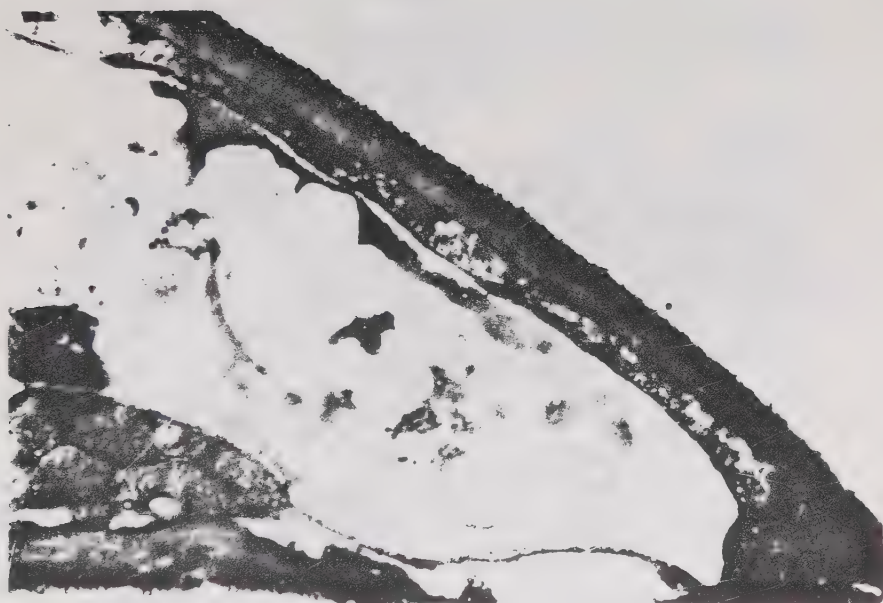


Fig. 16 a; Semithin epon sagittal section of Varroa female stained with Toluidin blue.

The section is showing the part of the opisthosoma in which the midgut coecum filled with host (Apis) hemolymph is occupying dorso-ventrally the hemocoel. Magnification: 500 x

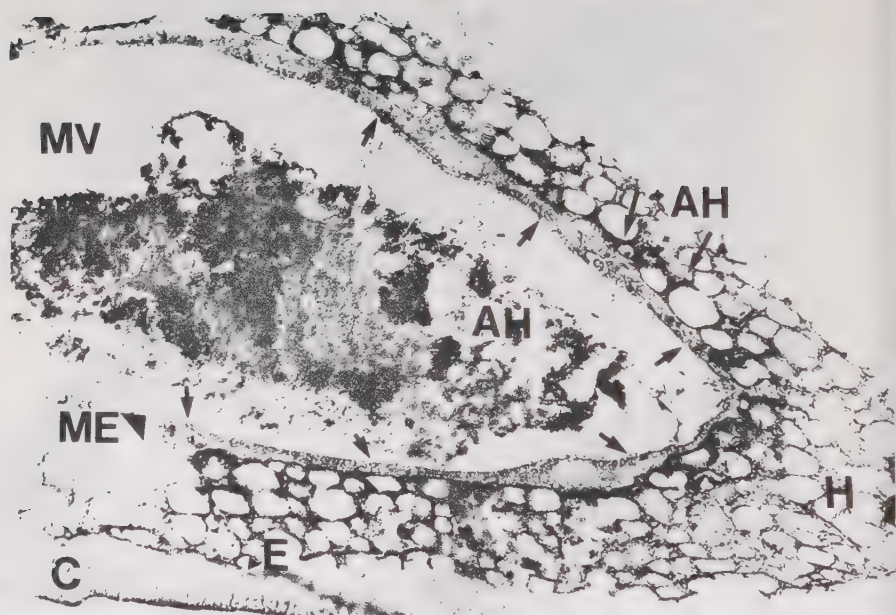


Fig. 16 b: Semithin section of the same mite as in Fig. 16 a but stained for unlabelled antibody-enzyme (PAP) method.

The black staining of the Varroa tissue shows the presence of Apis hemolymph proteins which are taken up without digestive degradation through the midgut epithelium. The bee antigens are labelled in Varroa tissue indirectly by using the antiserum (rabbit) directed against the bee hemolymph proteins. MV-midgut ventriculus; AH-Apis hemolymph proteins; H-hemocoel; C-cuticle; E-epidermis; ME-midgut epithelium. Magnification: 1000x.

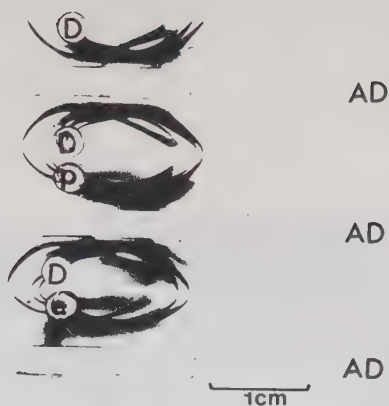


Fig. 17: Detection of Apis hemolymph proteins in the Varroa eggs. Immunoelectrophoresis in agarose gel. D-hemolymph of Apis drone larvae; P-hemolymph of pupae; e-Varroa egg homogenate; AD-antiserum directed against the Apis drone hemolymph mixture of larvae, prepupae and pupae. The patterns and full confluence of the precipitin arches are showing the identity of the antigens in various samples of bee and Varroa.

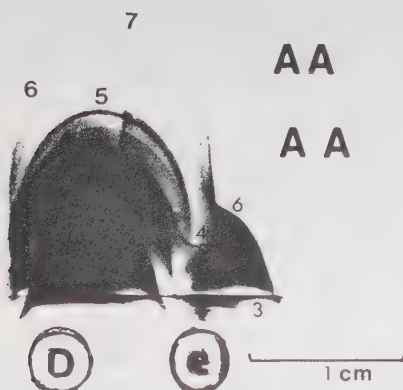


Fig. 18: Detection of Apis hemolymph proteins in the Varroa eggs. Two dimensional Tandem-cross-immunoelectrophoresis in antiserum impregnated agarose gel. D-hemolymph of Apis drone pupae; e-Varroa egg homogenate; AA-antiserum against Apis worker prepupae. Big numbers are for bee hemolymph proteins and small numbers for the mite's egg homogenate proteins. Protein fraction 3, 4, 6 in both samples are identical, producing double peaks.

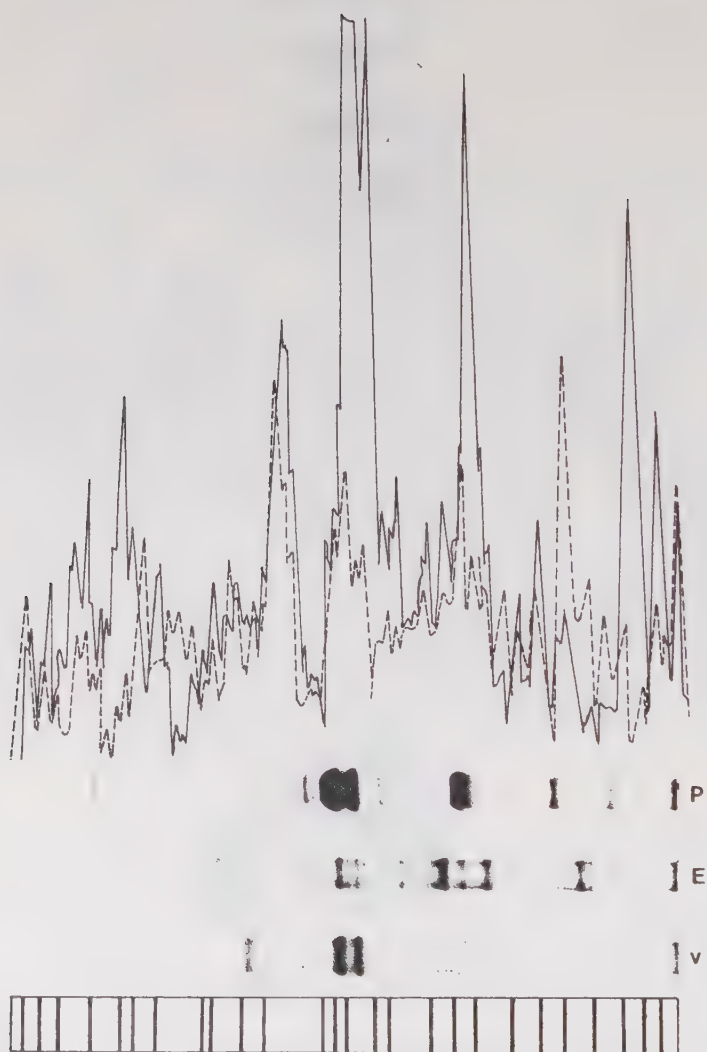


Fig. 19: Scanning pattern of 6 % SDS-PAGE, only *Apis* pupal hemolymph and *Varroa* egg homogenate samples are compared. Superimposition of about 20-25 proteinpeaks in the scanning patterns illustrate their identity. These bee proteins could only be resorbed into the mite eggs during the oogenesis from the hemolymph. (—) hemolymph from *Apis* pupae, (---) egg homogenate of *Varroa*.

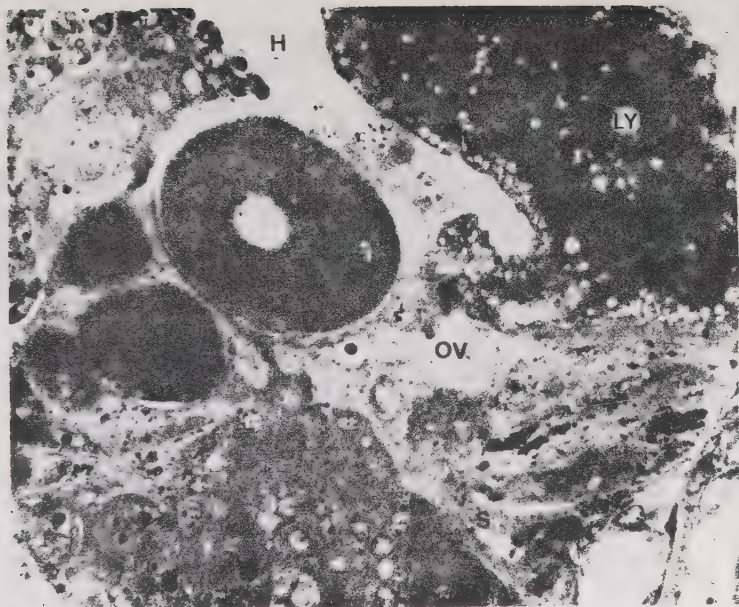


Fig. 20: Semithin cross section of Varroa female stained with Toluidine blue, showing ovary and surrounding tissues. C-cuticle; F-fat body; H-hemocoel; LY-lyrate organ; MV-midgut ventricle; OV-ovary; S-nutritive stroma (degenerating oocytes); ST-spermatheca. Magnification: 600 x.

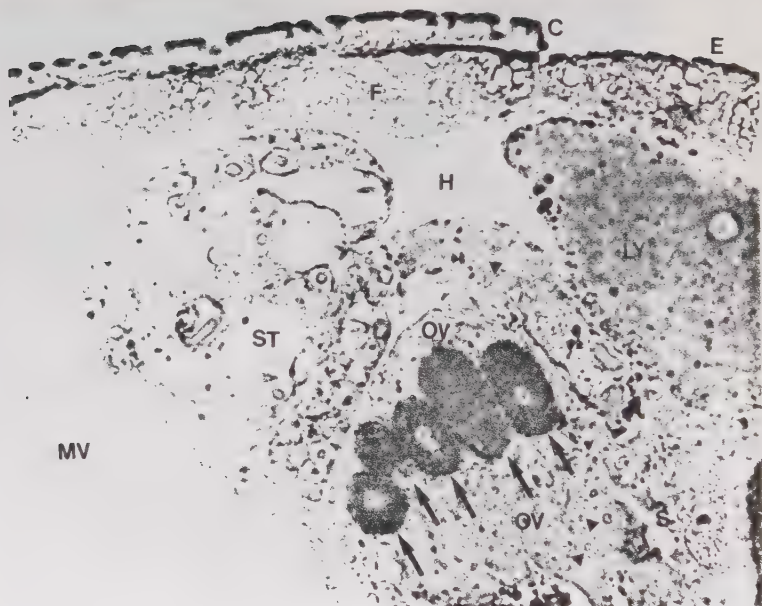


Fig. 21: Semithin cross section of the same mite as in Fig. 20 stained with unlabeled antibody-enzyme method. Legends as before. Small oocytes remained unstained (▶); heavily stained are the medium oocytes (→) Darkly stained oocytes indicate the presence of Apis hemolymph proteins. Magnification: 400x.

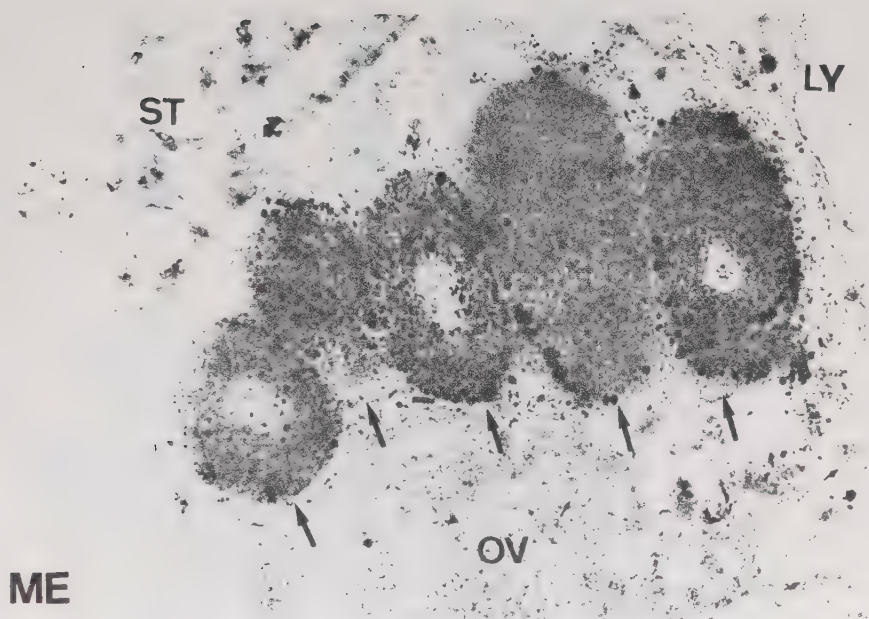


Fig. 22: Unlabelled antibody-enzyme method, same section as in Fig. 21 here enlarged,
Legends as in Fig. 21. Magnification 1250 x.

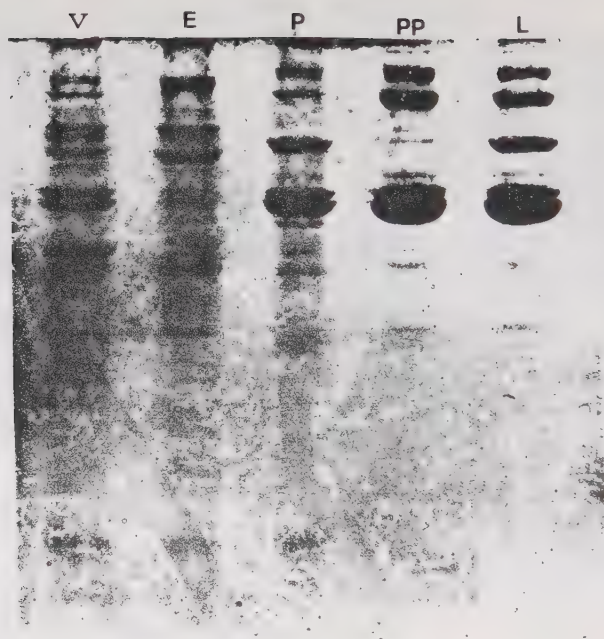


Fig. 23 a: SDS-polyacrylamide 15 % gel electrophoresis, Laemmli (1970) with discontinuous buffer system. v-hemolymph of Varroa female; P-pupal hemolymph; pp-prepupal hemolymph; L-larval hemolymph of Apis worker nursing stage bees.

A-F Apis hemolymph proteins without the counterparts in the mites hemolymph, apparently degraded during the resorbition; 1-5 are the Varroa own hemolymph proteins. Rest of the proteins are identical in almost all the samples.

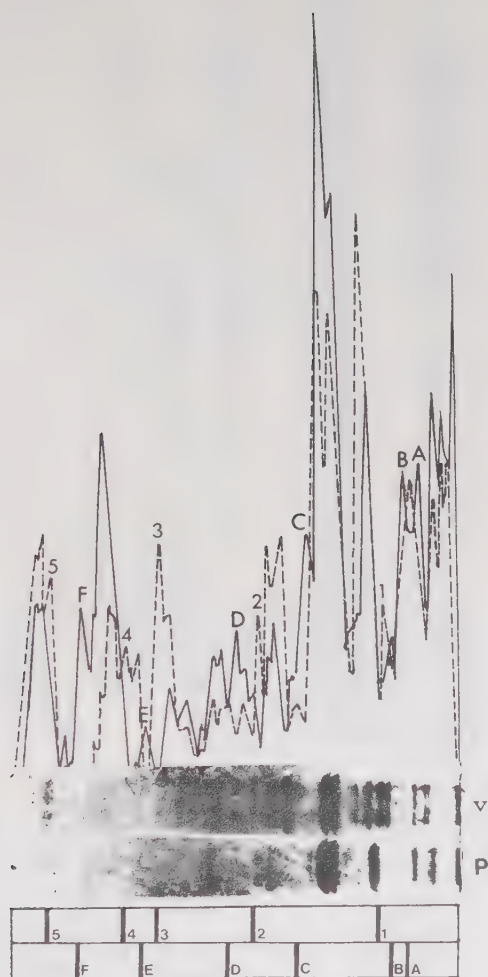


Fig. 23 b: Scanning pattern of the same gel as in Fig. 23 a.
 Only Apis pupal and Varroa female hemolymph samples are compared.
 Here also 1-5 are mites own proteins and A-F are bee proteins, which
 are absent in mite sample.
 (—) Apis pupal hemolymph; (-----) Varroa female hemolymph.

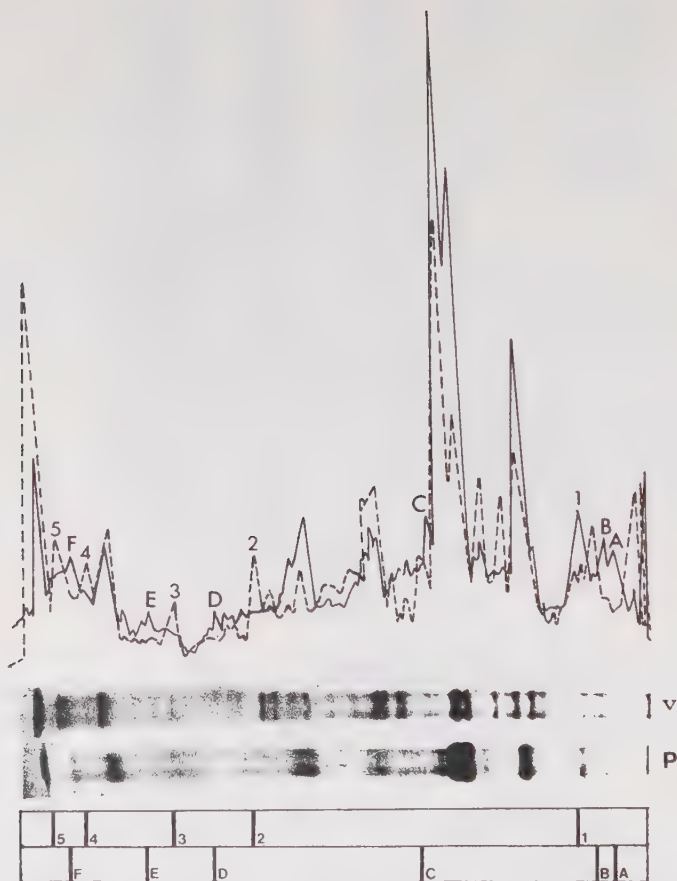


Fig. 23 c: Scanning pattern of 10 % SDS-PAGE, only Apis pupal and Varroa female hemolymph samples are compared. Explanation as in Fig. 23 b.

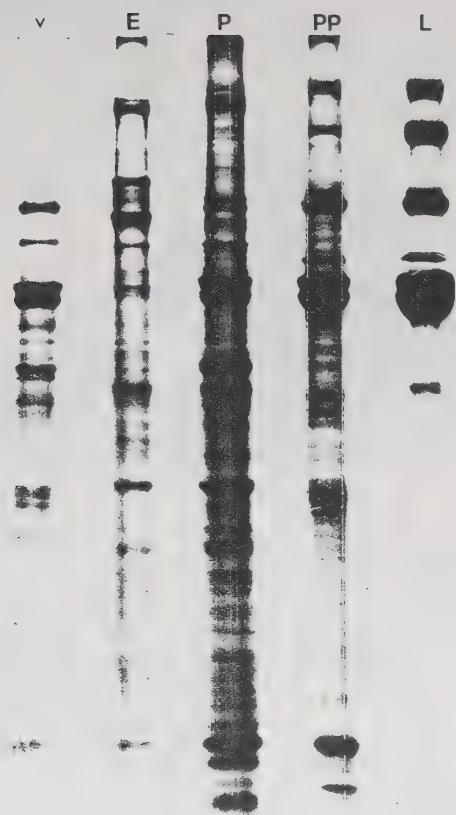


Fig. 24 a: Identification of own and host hemolymph proteins as vitellogenins of Varroa.

SDS-PAGE in 12 % slab gel. v-hemolymph of Varroa female; E-egg homogenate of Varroa; P-hemolymph of Apis pupae; PP-prepupal hemolymph; L-larval hemolymph.

1-8 (excepted no. 2, which is Varroa's own hemolymph protein) host originated proteins are accumulated in higher concentrations in the mites eggs more than its hemolymph, contrary to A-I, which are in higher concentration in the mites hemolymph. Therefore, 1-8 proteins are classified as 'vitellogenins' of Varroa females.

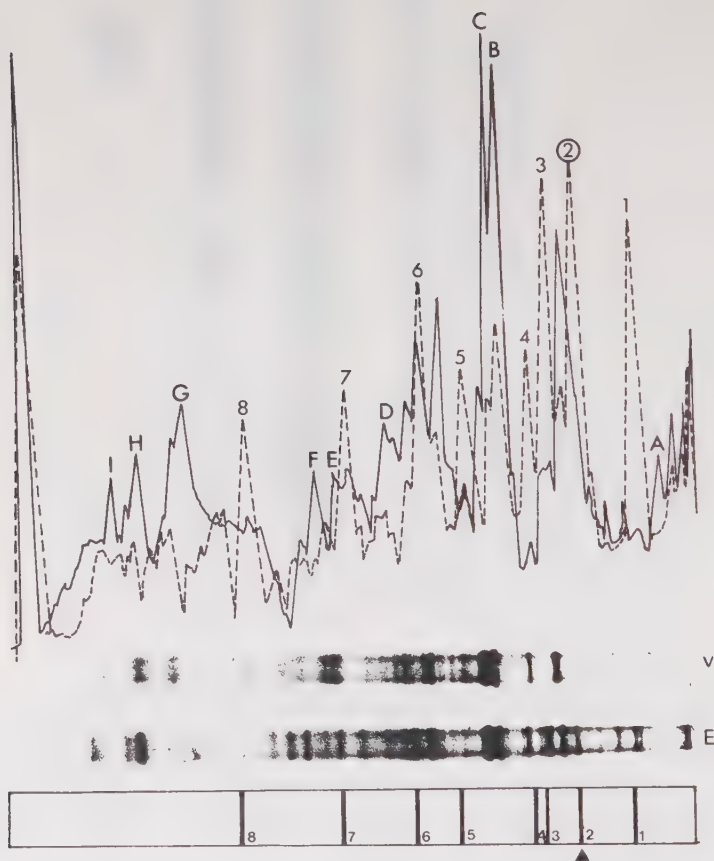


Fig. 24 b: Gel scanning pattern of the same gel as in Fig. 24 a. Here only *Varroa* females hemolymph and its egghomogenate samples are compared.
 (—) *Varroa* egg homogenate; (-----) *Varroa* female hemolymph sample. Explaination as before.

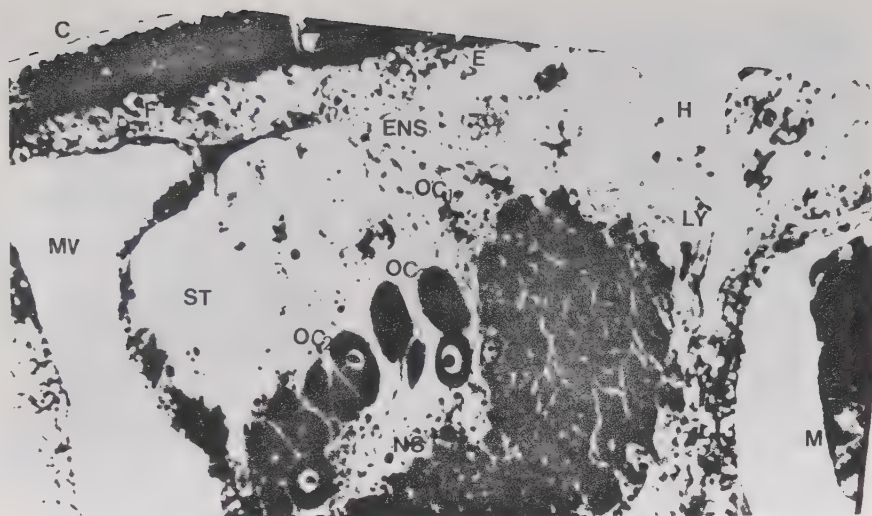


Fig. 25: Semithin epon cross section of female Varroa during the winter reproductive diapause, showing the ovary with several oocytes in phase 2. C-cuticle; E-epidermis; F-fat body; H-hemocoel; MV-midgut ventricle; M-muscles; ST-spermatheca; V-vagina; U-uterus; LY-lyrate organ; OV-ovary; OC-Oocytes; OC₁-degenerating Oocytes; OC₂-functional oocytes; OC₃-previtellogenic oocytes; NS-nutritive stroma; ENS-extraovarian nutritive cells. Magnification 500 x.

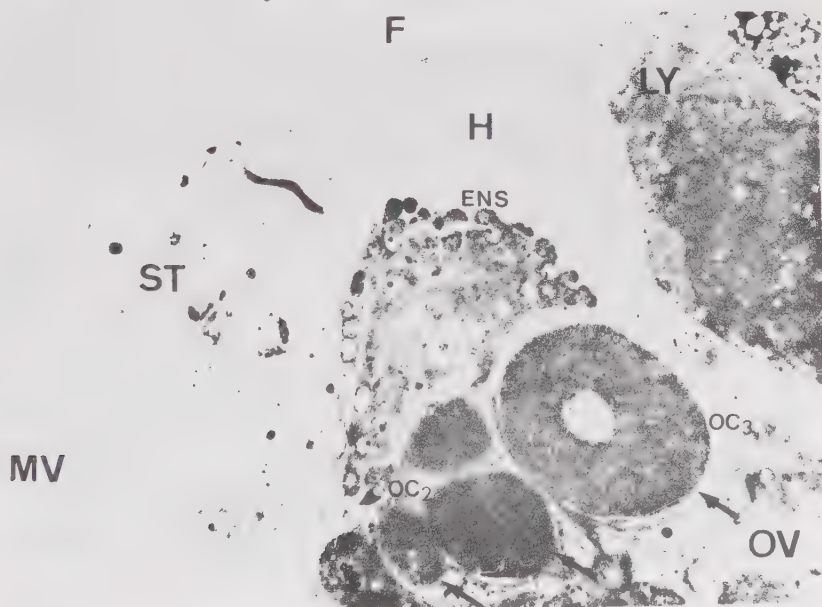


Fig. 26: Semithin epon cross section of female Varroa mite, during the beginning of summer reproduction phase. Explanation as above.

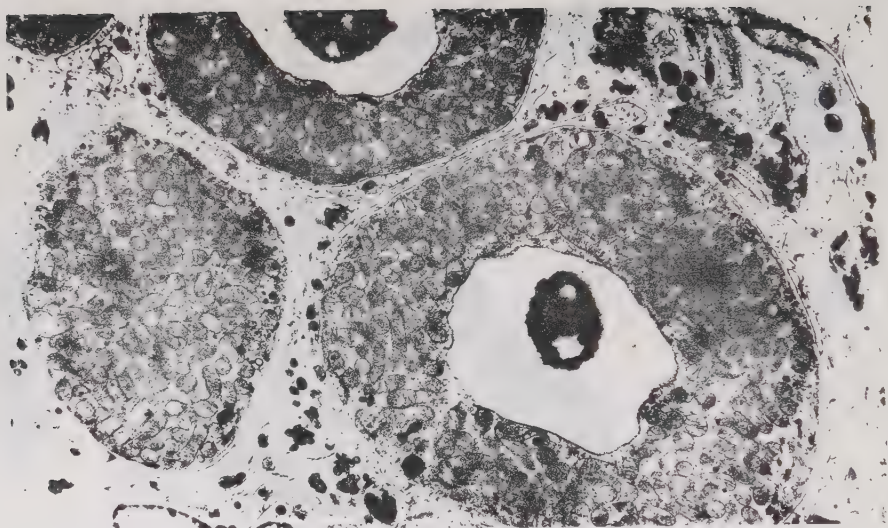


Fig. 27 a: Electronmicrograph (3000 x) of diapausing Varroa oocytes showing the arrested development of oocytes. The oocytes are fully packed with mitochondria but no yolk deposition. The oocytes are surrounded by endothelial cells whose nuclei are visible on the left corner.

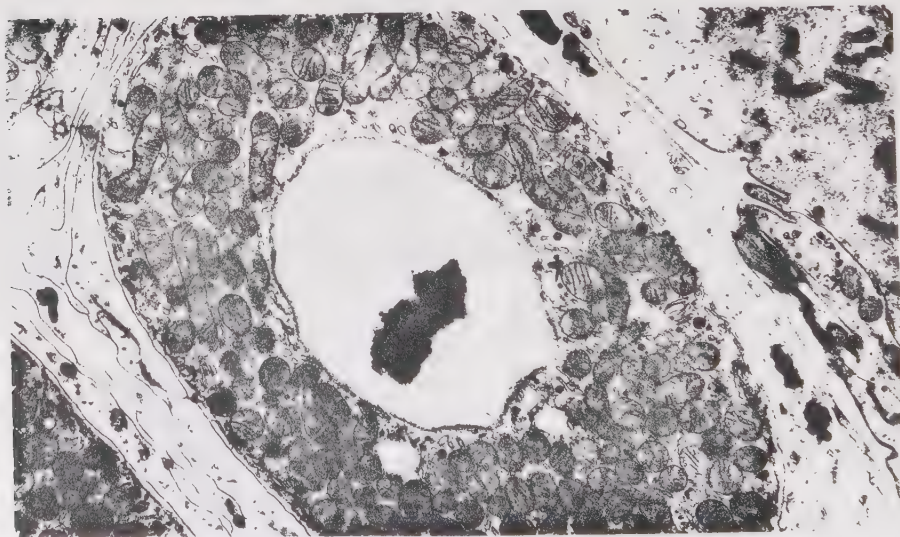


Fig. 27 b: Electronmicrograph (5000 x) of the same stage mites as above. Oocytes have branching mitochondria, condensed chromatin material and porous nuclear membrane.

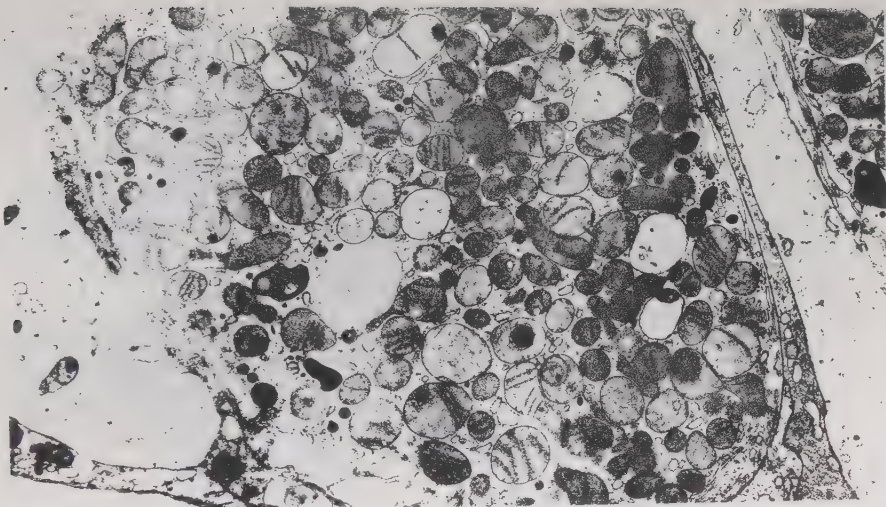


Fig. 27 c: Electronmicrograph (6000 x) of the winter diapausing oocytes. Few degenerating mitochondria, vesicles filled with electron dense material arising inside few of them and empty vesicles are visible.

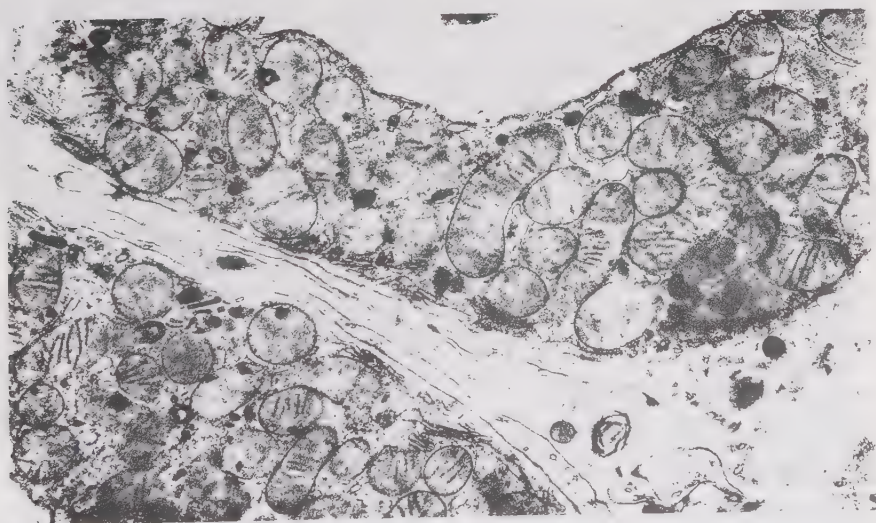


Fig. 27 d: Electronmicrograph (10 000 x) of the same stage mite. Under the oolemma golgi zones, dictysomal association with vesicles, and vesicles also in juxtaposition to nuclear membrane.

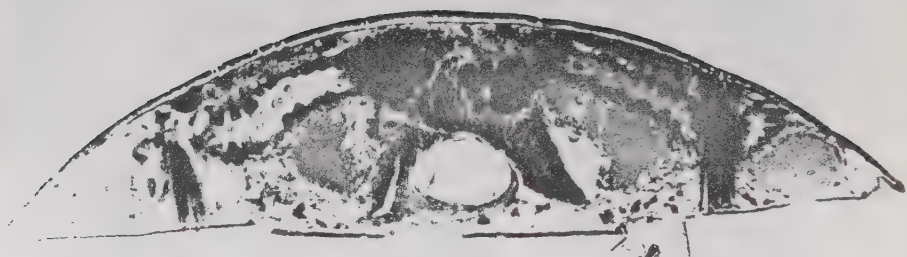


Fig. 28 a: Semithin epon cross section of female Varroa mite, showing the internal anatomy. The ovary contains oocytes in different stages of development along with extended lyrate organ. Magnification 130 x.

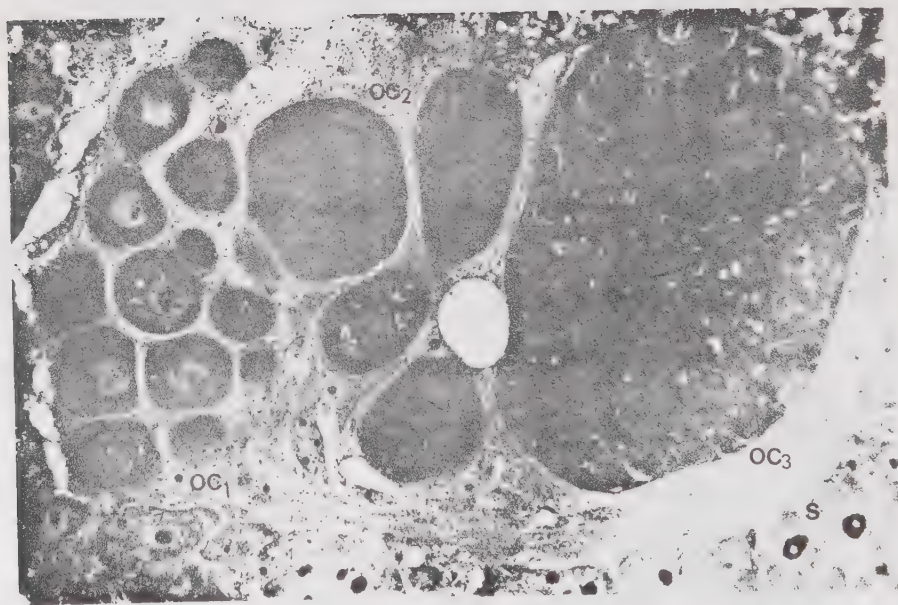


Fig. 28 b: Enlargement of the Fig. 28 a. showing only the ovary region with oocytes in different stages.

OC₁-functional oocytes of first order; OC₂-oocytes of second order; OC₃-vitellogenic oocytes; S-nutritive stroma (degenerating oocytes). Magnification 1200 x.

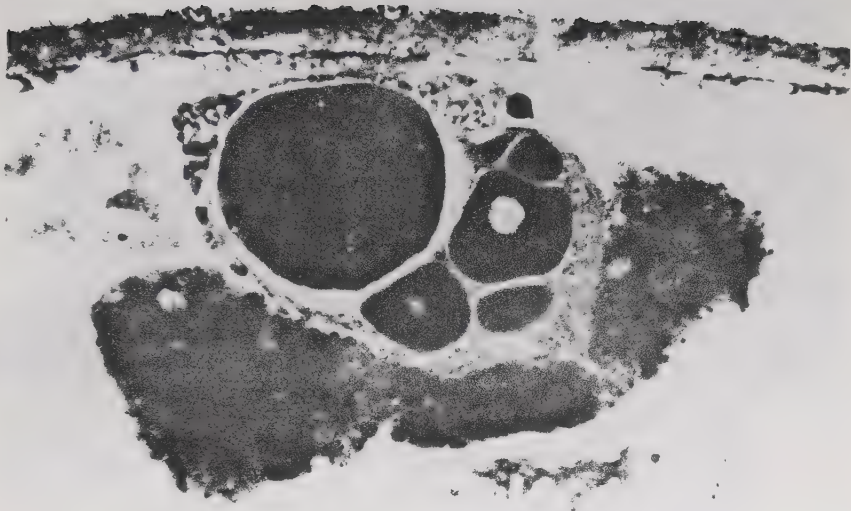


Fig. 29 a: Semithin epon cross section of Varroa female.

It shows the ovary and surrounding tissues. The ovary contains one big vitellogenic oocyte and 2 previtellogenic oocytes. Magnification: 400 x.

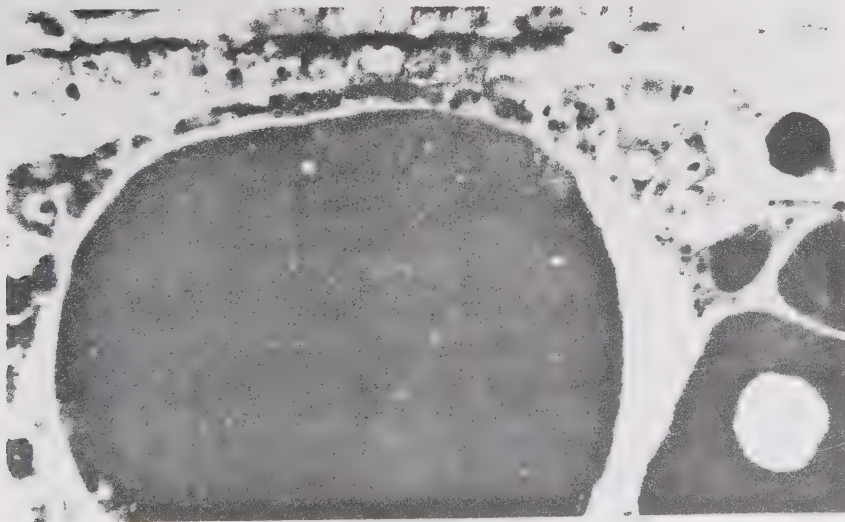


Fig. 29 b: Enlargement of the Fig. 29 a: The vitellogenic oocyte is surrounded by extraovarian nutritive cells. The upper part of the oocyte is showing the yolk deposition. Magnification: 1500 x.

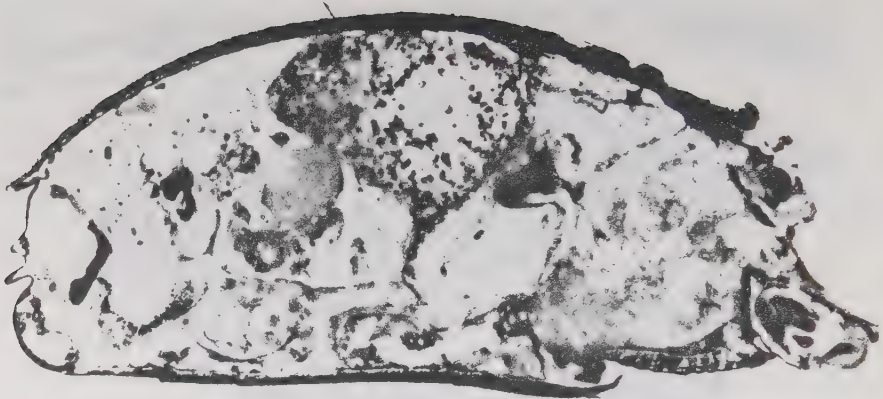


Fig. 30 a: Semithin epon sagittal section of Varroa female, showing the ovary, vitellogenic oocyte and egg. In the vitellogenic oocyte and the egg yolk deposition can be seen. Magnification 160 x.

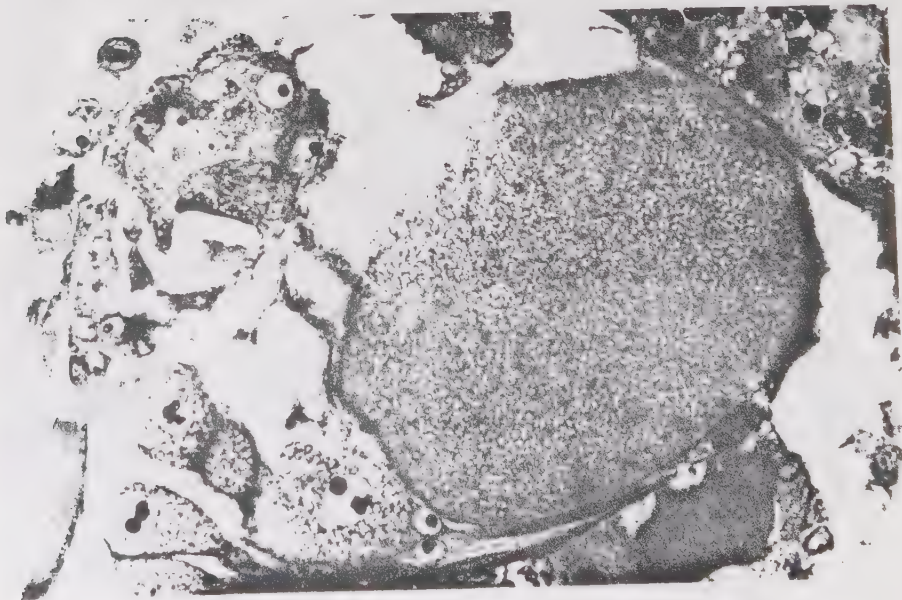


Fig. 30 b: Enlargement of the same section as in Fig. 30 a. The vitellogenic oocyte is packed with yolk spheres. Magnification 800 x.

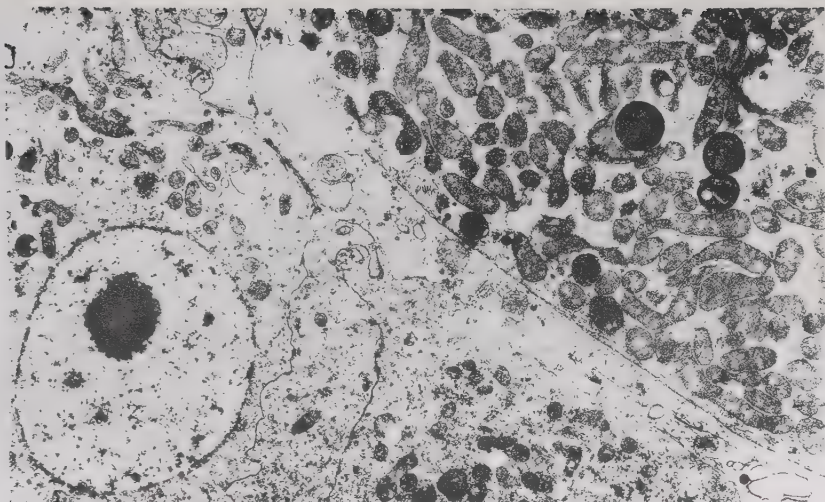


Fig. 31 a: Electronmicrograph (25 000 x) of the cross section of the female Varroa early previtellogenic oocytes and degenerating oocytes. Two types of oogonia near the margin of previtellogenic oocyte are visible, the oocyte with nucleus is a functional oocyte and adjacent to it are degenerating.

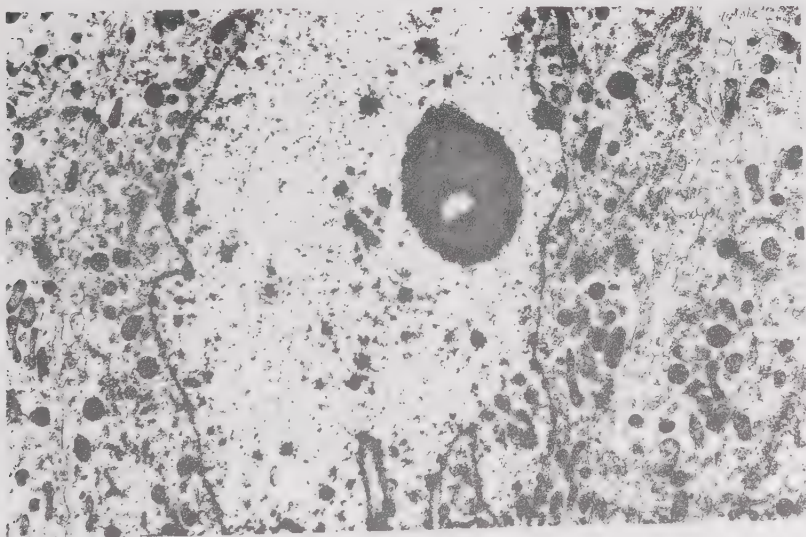


Fig. 31 b: Electronmicrograph (6 000 x) of cross section of Varroa females showing degenerating oocyte with karyolytic changes in oogonia, characterized by disappearance of oolemma and presence of pseudopodia like processes of the nuclei. The fragmented chromatin material is distributed all over.

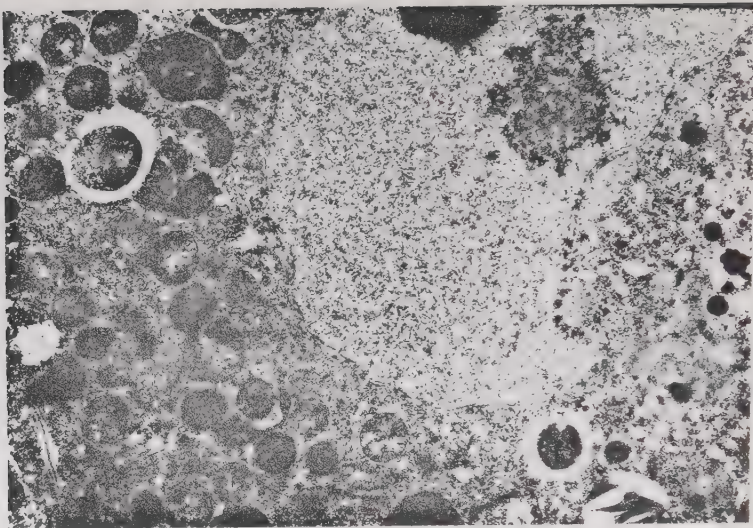


Fig. 32 a: Electronmicrograph (8 000 x) of cross section of Varroa female late previtellogenic oocyte, showing degenerating mitochondria, some yolk deposition, emission of electron dense material from nucleoplasm to ooplasm on the right side of the photo.

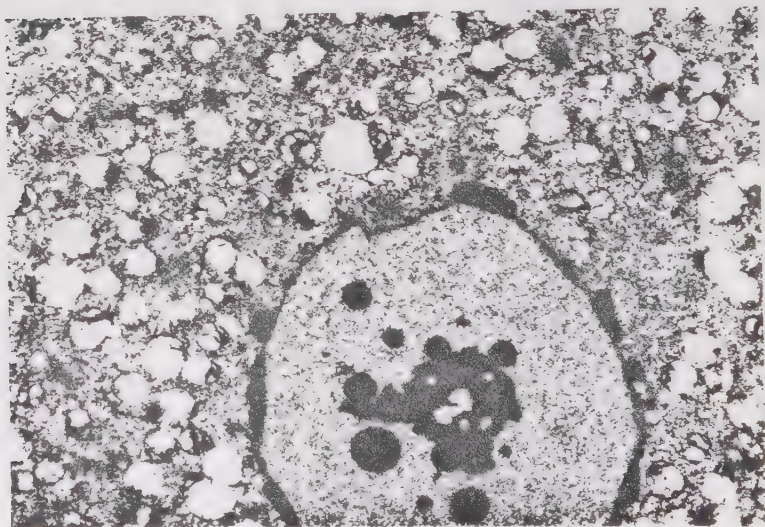


Fig. 32 b: Electronmicrograph (3 000 x) of a female Varroa previtellogenic second order oocyte. Mitochondria have disappeared. Electron dense material in juxtaposition to nuclear membrane.

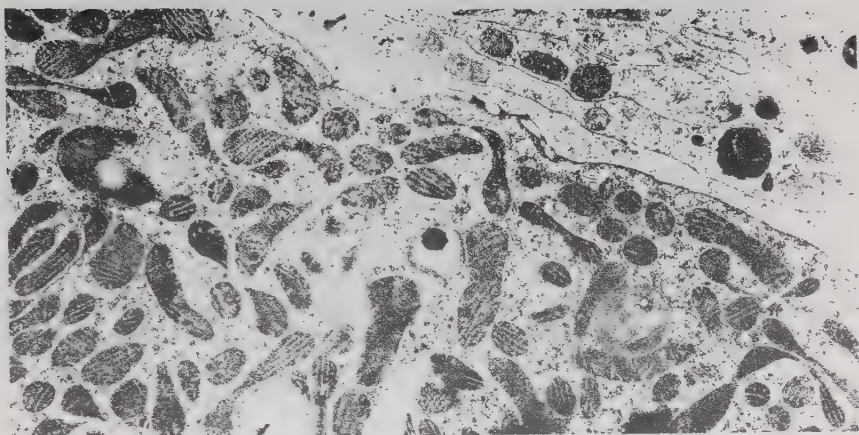


Fig. 33 a: Electronmicrograph (8 000 x) of the cross section of Varroa female early vitellogenic oocyte.

It shows some enfoldings of oolemma and degenerating mitochondria in the ooplasm.

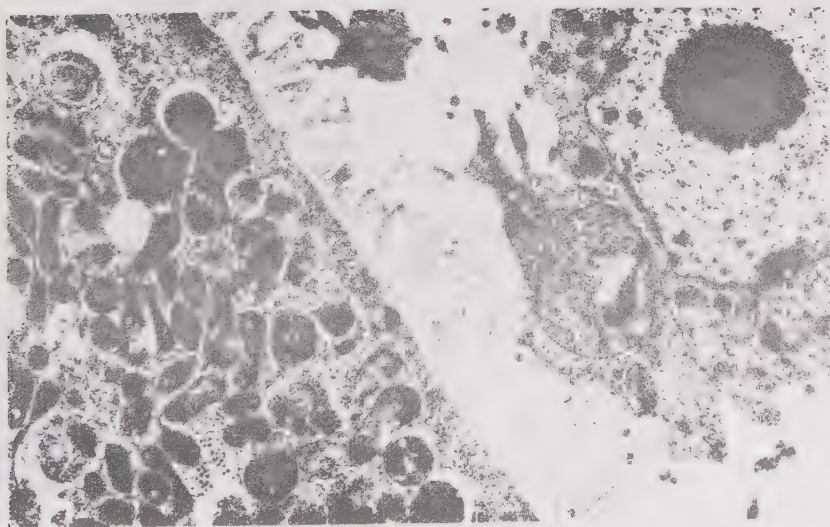


Fig. 33 b: Electronmicrograph (8 000 x) of Varroa females cross section passing through the vitellogenic oocyte in close association of hemocyte. The 'microlamellae' or specialized foldings of oolemma contain electron dense material identical to multivesicular bodies and yolk spheres.

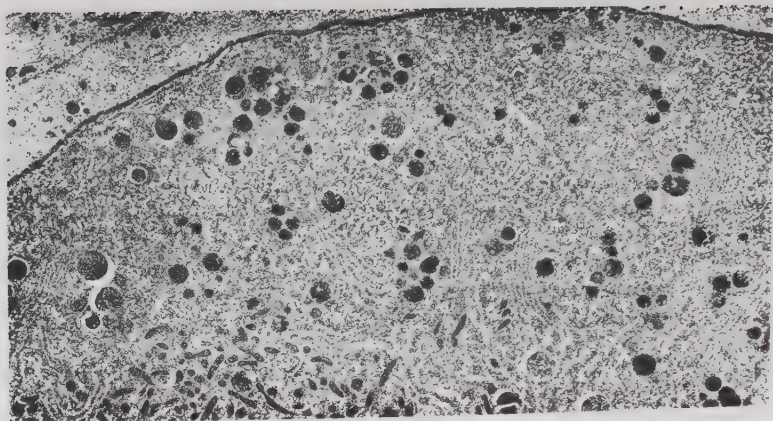


Fig. 34: Electronmicrograph (3 000 x) of cross section of Varroa vitellogenic oocyte.

Yolk uptake starting at one side of the oocyte. Degenerating mitochondria are still centrally situated, the ooplasm is rich in parallel rows of ER, yolk spheres are visible.

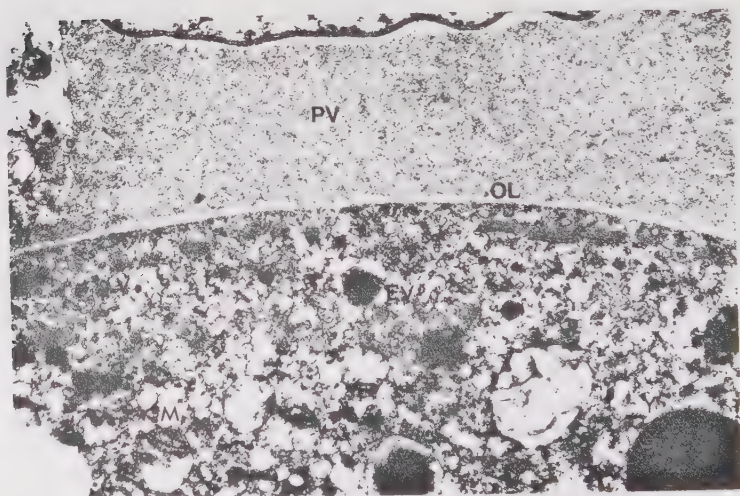


Fig. 35 a: Electronmicrograph (3 000 x) of cross section of Varroa vitellogenic oocyte, showing the endogenous egg shell or chorion building.

PV-perivitellin space; OL-oolemma; EV-endocytotic vesicle with yolk granules; V-vesicles transporting chorion building substance; Y-yolk spheres; M-degenerating mitochondria.

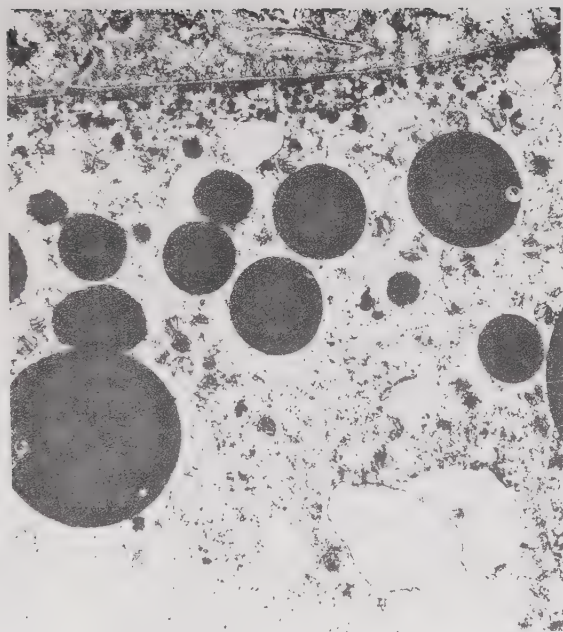


Fig. 35 b: Electronmicrograph (3 000 x) of cross section of Varroa late vitellogenic oocyte, showing the endogenous chorion deposition.

My academic teachers in Tübingen have been Profs. Drs.:

Bentrup, Engels, Hanack, Seyffert, Sperlich and Wöhrmann.

CURRICULUM VITAE

- 17.01.1947 Born in Upper Chopra, Pauri-Garhwal, U.P., India.
- 1953-1958 Attended Thoburn Primary School, Chopra Pauri; Garhwal, U.P., India.
- 1958-1963 Joined the school in Messmore Intermediate College, Pauri-Garhwal, India.
- May, 1965 Passed Intermediate Certificate Examination.
- 1965-1967 Studied Biology as main subject in Ewing Christian College, Allahabad, India.
- June, 1967 Passed B.Sc. (Bachelor of Science).
- 1967-1970 Post Graduate studies in Zoology with specialization in Entomology at University of Allahabad.
- June, 1970 Passed M.Sc. (Master of Science).
- 1970-1971 Taught Biology in Birla Public School, Nainital.
- 1971-1975 Taught Zoology as a lecturer in Ewing Christian Degree College, Allahabad, India.
- 1975-1977 Worked as a lecturer in Isabella Thoburn College, Lucknow, India.
- 1977-1978 Returned back to the same job in E.C. College.
- 1978- (on Stay in BRD.
leave)
- 1978-1980 German language course in the Studien Kolleg, Ökumenisches Studienwerk, Bochum. Since then scholarship holder of the same institute.
- 1980-1983 Ph.D. scholar with Prof. Dr. Wolf Engels in the Institute for Developmental Physiology, University of Tübingen. During the same period passed the Diplom Examination in Zoology, Botany, Genetics and Organic Chemistry.

